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CONTRACT NUMBER DAMD17-93-C-3146

TITLE: Quantitative Assessment of HIV Replication and Variation In Vivo: Relevance to Disease Pathogenesis and Response to Therapy

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REPORT DATE: December 1996

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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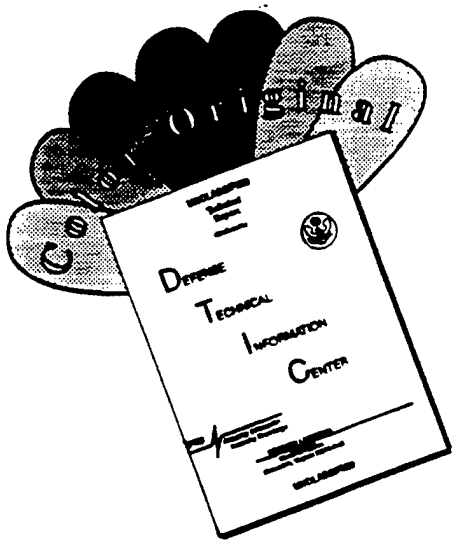

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1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1996	Final (22 Jun 93 - 31 Dec 95)

4. TITLE AND SUBTITLE Quantitative Assessment of HIV Replication and Variation In Vivo: Relevance to Disease Pathogenesis and Response to Therapy

5. FUNDING NUMBERS
DAMD17-93-C-3146

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The pathogenesis of HIV-1 disease is driven by continuous rounds of viral replication in lymphoreticular tissues. Plasma virus load is believed to reflect virus production in these tissue sites, but the dynamics and quantitative relationships between virus populations the blood and lymphoid tissues remain to be determined. In the studies described, we have developed quantitative approaches for evaluating plasma viral RNA content and composition. We show that plasma viral RNA is virion-associated, is correlated with plasma viral p24 antigen and infectivity titers, and can be accurately quantified by RT-PCR and branched DNA (bDNA) signal amplification assays. Furthermore, we show that initiation of potent antiretroviral therapy, or initiation of a cytotoxic T-lymphocyte (CTL) response in primary (acute) infection, leads to rapid declines in plasma virus load and replacement of wild-type virus populations by escape variants. Importantly, the studies show for the first time that virus-specific CTL exert a biologically significant suppressive effect on HIV-1 replication *in vivo*, comparable in magnitude to the effects of antiretroviral chemotherapy. Plasma virus was shown to have a half-life ($T_{½}$) of 6 hours, virus-producing lymphocytes a T½ of 2 days, and latently infected cells a T½ of 10-21 days. These data provide a quantitative and dynamic assessment of HIV-1 replication *in vivo* and provide insight into the biological activity of antiretroviral drugs and the host immune system in natural infection.

14. SUBJECT TERMS	15. NUMBER OF PAGES
AIDS, HIV-1, Viral Load, HIV-1 Pathogenesis, HIV-1 Vaccine, HIV-1 Drug Therapy	144
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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12/18/96

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"Quantitative Assessment of HIV Replication
and Variation *In Vivo*: Relevance to Disease
Pathogenesis and Response to Therapy"

INTRODUCTION

In July 1992, we proposed a five year research program aimed at characterizing HIV-1 load and expression in blood and tissues of infected humans and at characterizing the role of genetic and biologic variability of HIV-1 in disease pathogenesis. A contract was awarded for a three year period of study which was then shortened further to two years and three months. Despite this abbreviated period of funding, substantial progress was made, including the publication of seven manuscripts. Among these were a paper by Wei *et al.* ("Viral dynamics in HIV-1 infection" *Nature* 373:117-122, 1995) which has been recognized as having led to a paradigm shift in the understanding of HIV-1 pathogenesis and optimal strategies for its treatment and prevention. More recently, we have submitted and had accepted for publication a manuscript by Borrow *et al.* ("Antiviral pressure exerted by HIV-1-specific CTL during primary infection demonstrated by rapid selection of CTL escape virus" *Nature Medicine*, in press) which demonstrates for the first time that cytotoxic T-lymphocytes exert substantial antiviral pressure in acute (primary) infection, leading to the emergence of CTL escape variants in a manner comparable to viral drug resistence development. This finding, too, represents a major advance in our understanding of HIV-1 pathogenesis and has important implications for vaccine development. In other studies, still underway, we are defining the frequency and lifespans of blood and lymphoid cells that harbor latent, defective, and actively replicating viral genomes.

BODY

Persistent viral replication is now recognized for its central role in human immunodeficiency virus type 1 (HIV-1) pathogenesis and natural history (1-8). Viral load determinations in plasma, peripheral blood mononuclear cells (PBMCs), and lymphoid tissue have been significantly correlated with clinical stage and $CD4^+$ lymphocyte counts, with highest viral levels occurring in primary (acute) and late stage infection and lower levels in early and intermediate stages (1-13). Certain viral measurements such as p24 antigen (p24

Ag) have been shown to have prognostic value (14, 15), but their utility as surrogate markers for clinical endpoints in therapy trials remains to be fully proven (16, 17).

A number of different viral markers have been pursued as potentially valuable clinical indicators of disease activity as well as for surrogate markers of clinical endpoints (reviewed in 16). These include quantitative viral cultures of PBMCs and plasma (1-3); p24 Ag and immune complex dissociated (ICD) p24 Ag (13-15, 17-21); and polymerase chain reaction (PCR) amplification of PBMC-associated viral DNA and RNA (22-26). All of these markers are directly linked to the underlying HIV disease process and thus could be expected to provide clinically relevant information. Yet, each of these assays has significant theoretical or practical limitations. For example, PBMCs comprise only a small proportion (≈2%) of total lymphoid tissue (27) and may contain transcriptionally latent or defective provirus (23-32). When stimulated in culture, cells expressing viral proteins are targeted for destruction or suppression by autologous HIV-specific T-lymphocytes (33,34). Quantitative virus cultures of plasma are similarly compromised as a viral load measurement by a high and variable proportion of defective virus (8) and by the effects of neutralizing antibodies (35). Furthermore, quantitative cultures are costly, time-consuming, and associated with substantial exposure to infectious virus. Another viral marker, p24 Ag, is released from cells either as a virion component or as non-virion-associated antigen (36, 37). p24 measurements thus reflect a combination of virus production, provirus expression, and even p24 Ag release from dying cells. Moreover, p24 Ag is often complexed with circulating anti-p24 antibody and is only variably detected even by the ICD-p24 Ag assay (16, 18, 19).

Recently, there has been growing recognition of plasma virion-associated RNA as a direct, sensitive, and quantifiable measure of viral load (8, 9, 23, 38, 39). In theory, plasma viral RNA should reflect virus production throughout the entire lymphoreticular system rather than the comparatively minor PBMC compartment. Viral RNA determinations are not affected by the biologically complex requirements of virus culture and propagation *in vitro* nor by the variabilities associated with p24 Ag production and measurement.

A number of different PCR, T7 RNA polymerase, and branched DNA signal amplification assays for quantifying viral RNA in plasma have been developed (8, 9, 23, 38-51). Preliminary evaluation of these assay methods has generally yielded comparable data indicating that plasma viral RNA can be detected in most infected subjects, that levels are related to clinical stage and CD4 counts, and that institution of antiretroviral therapy results in declines in viral RNA.

The Quantitative Virology Working Group of the NIH/NIAID AIDS Clinical Trials Group (ACTG) recently conducted a blinded, multicenter evaluation of six different plasma viral RNA assays [Chiron bDNA assay (49); Roche RT-PCR assay (44); Abbott immunocapture PCR assay (50); and three noncommercial RT-PCR assays (9, unpublished data)] and concluded that several of them exhibited the requisite sensitivity, specificity, and precision to proceed with their evaluation in clinical trials (39). Despite the introduction of the bDNA assay and other commercially available assays for HIV-1 RNA quantitation into clinical trials, scant correlative information has been published describing the relation of HIV-1 RNA levels determined by these assays with respect to clinical stage, $CD4^+$ counts, other conventional virologic markers, or response to antiretroviral therapy. Moreover, no studies have been published directly comparing two independent HIV-1 RNA assays using large numbers of well-characterized clinical samples as a means to cross-validate the respective methodologies. The following work was thus designed to evaluate the results of QC-PCR (RT-PCR) and bDNA assays in patients representing the complete clinical spectrum of HIV-1 infection; define the quantitative relationship between viral load measurements determined by QC-PCR and bDNA assays and those determined by quantitative plasma culture and regular and ICD-p24 Ag analysis; determine the magnitude and kinetics of change in QC-PCR and bDNA determined viral RNA levels compared with other viral markers in patients treated with zidovudine; and evaluate changes in virus load, viral genotype, and viral phenotype following the initiation of potent antiretroviral therapy with non-nucleoside reverse transcriptase (RT) or protease inhibitors and in the setting of acute (primary) infection. The objectives of these latter studies were thus to determine the dynamics of virus turnover in plasma and cellular

compartments in response to virus suppression by antiretroviral drugs or the body's immune cytotoxic T lymphocyte (CTL) response.

The current contract work entitled "Quantitative Assessment of HIV Replication and Variation *In Vivo*: Relevance to Disease Pathogenesis and Response to Therapy" (DAMD17-93-C-3146) addresses the following hypotheses: (i) HIV replication and expression, tissue burden, and genetic and biologic variability are viral parameters that are inseparably linked and which are directly related to the pathogenesis of AIDS, and (ii) Elucidation of viral pathogenesis and development of effective treatments and vaccines for HIV will be facilitated by better quantitative measures of virus replication *in vivo* and by experimental models that relate these measures of virus replication to clinically important changes in the viral envelope and reverse transcriptase genes. The specific aims that we have addressed are as follows:

1. To determine the biological and biophysical relationships between novel measures of HIV-1 replication and expression *in vivo* and to determine the potential value of these virologic parameters as surrogate markers for clinical endpoints in natural history and therapeutic interventional trials.

2. To determine the rate and molecular characteristics of genetic change in HIV-1 envelope and reverse transcriptase (RT) genes in uncultured, PCR amplified strains of HIV-1 from patients with acute and early chronic infection (CDC stages I and II) and from later stage patients who have been treated with potent non-nucleoside RT inhibitors and have developed drug resistance. The goal of this aim is to determine the relationship between rates of viral replication measured by HIV-1 RNA, DNA, p24 Ag, and infectious virus production (specific aim #1) and the accumulation of genetic changes in specific viral genes whose products are under selective pressures *in vivo*.

3. To determine the biological and clinical consequences of HIV-1 genomic variation identified in aim #3 in regard to envelope function and antigenicity. The goal of this aim

is to determine the pathogenic significance of these envelope changes by characterizing their biologic properties in the genetic background of biologically relevant proviral clones representing T-cell and monocyte tropic viruses that were obtained in the current contract period.

Materials and Methods

Patients. For the quantitative analysis of HIV-1 RNA and p24 antigen in peripheral blood, specimens were obtained from a total of 271 study subjects. At the Aaron Diamond AIDS Research Center (ADARC), blood was collected from 80 randomly selected HIV-1 seropositive individuals seen at New York University Medical Center, the New York Blood Center, and Bellevue Hospital. The subjects included patients with acute HIV-1 infection (2 cases), AIDS (3 cases), ARC (10 cases), and asymptomatic illness (65 cases). History of antiretroviral therapy in these individuals was unknown. Plasma specimens obtained from 90 HIV-1 seronegative subjects served as controls. Plasma samples (collected in heparin) were prepared from blood specimens that were centrifuged at 400 X g for 10 minutes at room temperature and were stored at -80°C until analysis. At UAB, plasma specimens for bDNA analysis were obtained from archived samples remaining from two previous clinical trials (8, 52). In the first instance, replicate frozen (-70°C) plasma samples from each of 60 subjects previously evaluated for HIV-1 plasma viral RNA by the QC-PCR method in a study of HIV-1 natural history (8) were analyzed. In the second instance, replicate plasma samples from 12 subjects representing the zidovudine (azidothymidine, AZT) control arm of a prospective, randomized, double-blinded phase I/IIA study of the non-nucleoside analogue reverse transcriptase inhibitor L-697,661 (52, 53) were examined. In both cases, the samples culled for bDNA analysis were unselected and represented the entire sample sets for the respective studies. The 72 UAB study subjects included 6 patients with acute infection, 25 with asymptomatic infection, 24 with ARC, and 17 with AIDS. Twenty-nine control samples were obtained from healthy, uninfected volunteers. Plasma specimens at UAB were derived from blood samples that had been collected in acid citrate dextrose (ACD) and processed by sequential 15 minute

centrifugations at 200 x g and 1000 x g prior to storage at -80°C in order to ensure the removal of the majority of platelets. In addition to the cross-sectional clinical samples, sequential plasma specimens were collected from two ADARC patients with primary (acute) infection and from 12 UAB patients who were begun on a six week course of zidovudine (52, 53). For the analysis of viral dynamics following initiation of drug therapy or in acute infection, 23 additional subjects were selected for study, as described subsequently.

p24 Antigen Assay, Quantitative Culture, and QC-PCR. Plasma p24 antigen levels were determined by commercial enzyme immunoassays (ADARC-Abbott Laboratories, Inc., N. Chicago, IL; UAB-Coulter Diagnostics, Hialeah, FL). Sensitivity cutoff values for the p24 antigen assays were 5 pg/ml for the regular assay and 15 pg/ml ICD method. The quantitation of infectious HIV-1 titers in plasma was performed using the end-point-dilution culture method as described previously (1, 3). Levels of HIV-1 RNA in plasma for all UAB specimens were determined by the QC-PCR method (8). QC-PCR, infectious plasma virus titers, and p24 antigen results for the UAB subjects have been reported previously (8, 53).

bDNA Assay. HIV-1 RNA in plasma was quantified at the ADARC and UAB study sites using the branched DNA signal amplification method (Quantiplex™ HIV-RNA Assay, Chiron Corporation, Emeryville, CA) (45, 49). All plasma specimens were coded and blinded to individuals performing the assay and recording the assay results. Virus was concentrated from duplicate plasma specimens as recommended by the manufacturer by centrifugation in a bench top microcentrifuge (Heraeus Centrifuge Model 17RS, rotor 3753) at 23,500 g for 1 hr. Because of limited sample volumes, 0.25-1.0 ml specimens were initially used, adjusting their total volume as necessary to 1.0 ml with normal human plasma, and correcting the final bDNA readout by the corresponding dilution factor. This approach was shown experimentally not to affect the quantitative results of the bDNA assay except for its threshold sensitivity (unpublished data). For specimens yielding a negative bDNA result and for which less than the recommended duplicate 1.0 ml plasma volumes were initially tested, the assay was repeated using 1.0 ml samples. The virus pellet was

extracted in a buffer containing proteinase K, lithium laurel sulfate, and target probes complementary to the HIV-1 *pol* gene. This was followed by a 15-minute centrifugation step at 23,500 g, after which the supernatant was transferred to microwells (in a 96 well plate) coated with capture probes. By this means, the RNA-target probe complexes were captured onto the surface of microwells during an overnight incubation. The wells were washed and bDNA amplifier molecules were hybridized to the immobilized target-probe complexes, followed by hybridization of multiple alkaline-phosphatase-labeled probes to each bDNA molecule. The complexes were then incubated with a chemiluminescent substrate (dioxetane), and light emission was measured in a luminometer. The light emission was directly proportional to the amount of HIV-1 RNA present in the plasma specimen. The concentration of RNA in a specimen, expressed as HIV RNA equivalents (Eq) per ml of plasma. Samples containing less than 10^4 to 1.6 x 10^6 RNA Eq/ml of plasma. Samples containing less than 10^4 RNA Eq/ml were recorded as negative and those exceeding 1.6 x 10^6 were diluted 1:10 or 1:100 in negative plasma and re-assayed.

Viral RNA (cDNA) Sequence Analysis of Reverse Transcriptase. Mixtures of wild-type and mutant cDNA clones were prepared and diluted such that first round PCR amplifications were done with 1,000 viral cDNA target molecules per reaction. HIV-1 RNA was isolated from virions pelleted from uncultured plasma specimens as described (54). cDNA was prepared using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) and an oligonucleotide primer corresponding to nucleotides 4283 to 4302 of the HXB2 sequence. The full-length viral reverse transcriptase gene (1680 bp) was amplified by means of a nested PCR using conditions and oligonucleotide primers (outer primers: nt 2483-2502 and 4283-4302; inner primers: nt 2549-2565 and 4211-4229). Subgenomic fragments of the RT gene were also amplified using combinations of the following oligonucleotide primers: (5') 2585-2610; (5') 2712-2733; (3') 2822-2844; (3') 3005-3028; (3') 3206-3228; (3') 3299-3324; (3') 3331-3350; (3') 3552-3572; and (3') 3904-3921. All 3' primers incorporated the universal primer sequence for subsequent dye-primer sequence analysis. The HIV-1 copy number in every PCR reaction was determined (100-10,000 copies). A total of three to six

separate PCR amplifications of primary patient material was done on each sample using different combinations of primers. For sequencing, an automated ABI 373A sequenator and the Taq Dye Primer Cycle Sequencing Kit (ABI) were used. Sequences were analyzed using Sequencher (Gene Codes Corp.) and Microgenie (Beckman) software packages, and base pair mixtures were quantified by measuring relative peak-on-peak heights.

Viral RNA (cDNA) Sequence Analysis of Envelope gp160. The gp160 sequence of the autologous virus from patient WEAU 15 DFOSx was determined by automated DNA sequencing of the WEAU 1.60 provirus and is reported as the HIV-1 reference sequence in the 1995 HIV Molecular Immunology Database. Quantitative detection of gpl60 mutations occurring in the uncultured plasma virus population over time was performed as described (54). HIV-1 RNA was isolated from virions pelleted from plasma specimens, and cDNA prepared using antisense oligonucleotide primers corresponding to either 5'-TTGCTACTTGT GATTGCTCCATGT-3' (nt 8920-8943), 5'-TCTTATGAGTGTGGTGACATTGAAAGA-3' (nt 6706-6732), or 5'-CAGAGTGGGGTTAATTTACACATGG-3' (nt 6571-6596) (numbering according to HIV-1 proviral clone WEAU 1.60). Full length and partial gpl60 gene sequences were amplified by nested PCR as described using the primers listed above along with primers at positions 5851-5875, 5956-5981, 6203-6326, 6436-6455, 6571-6596, and 8889-8911. Primers incorporated the universal M13 sequence for subsequent dye-primer sequence analysis of the gpl60 aa 30-39 region. For sequence analysis of the complete gpl60 gene, dye-labeled dideoxynucleotide terminators were used. A total of 4-6 separate PCR amplifications of plasma viral RNA/cDNA (750 molecules/reaction) was done for each timepoint. Double strand sequence analysis was performed using an automated ABI 373A sequenator and Dye Primer/Dye Terminator Cycle Sequencing Kits (ABI). Sequences were analyzed using Sequencher (Gene Codes Corp.) and Microgenie (Beckman) software packages, and base-pair mixtures were quantitated by measuring relative peak-on-peak heights. Relative proportions of wild-type and mutant sequences were determined independently by subcloning the amplified gpl60 envelope products (above) into pCR3 (Invitrogen Corporation, San Diego,

CA) and subjecting the individual clones to double-stranded automated DNA sequencing.

Recombinant vaccinia viruses. Recombinant vaccinia virus vSC8, which expresses only *Escherichia coli ß* galactosidase; vPE16, which expresses gpl60 from HIV-1 IIIB; vCF21, which expresses Pol from HIV-l HxB2; vTFnef2, which expresses Nef from HIV-1 NL43 and vtat, which expresses Tat from HIV-1 IIIB, were obtained from Dr. Bernard Moss (NIH, Bethesda, MD); and recombinant vaccinia virus vAbT 141-5-1, which expresses the full-length p55 Gag protein from HIV-1 IIIB was obtained from Drs. Dennis Panicali and Gail Mazzara (Therion Biologics Corporation, Cambridge, MA).

Recombinant vaccinia viruses expressing full-length gpl60 (vM12) or sections thereof (vM1 and vM9) derived from the autologous HIV-1 in patient WEAU 15 DFOSx were produced by homologous recombination into the thymidine kinase gene of vaccinia virus using the vaccinia transfer plasmid pNVV3, a modified version of pSC 1 1, as described. The clone expressing full-length gpl60 used for the construction of the recombinant vaccinia viruses was obtained from a full-length replication-competent proviral clone (1.60) derived by lambda phage cloning of an isolate obtained 15 DFOSx. The envelope gene was subcloned into plasmid pCR II (Invitrogen Corporation, San Diego, CA), from which it was subsequently excised and ligated into the Sma 1 and Not 1 sites of pNVV3 to yield pM12. Sections corresponding to nucleotides (nt) 1-330 and 334-2580 of the *env* gene were amplified from the full-length clone by PCR using oligonucleotide primers which incorporated the necessary start and stop codons as well as an Sma 1 site at the 5' end and a Not 1 site at the 3' end. The PCR products were then ligated into pNVV3 to yield pMl *(env* ntd 1-330) and pM9 *(env* ntd 334-2580). pM12, pMl and pM9 were used to produce recombinant vaccinia viNses vM12, vMl and vM9, respectively. Protein expression was confirmed by Western blotting (not shown).

CTL assays. Cryopreserved PBMC were expanded *in vitro* by bulk culture for 10 days. For use in other experiments, CTL lines/clones were established from PBMC cryopreserved from patient WEAU 20 DFOSx by culture at limiting dilution. These were operationally referred to as clones. CTL activity was

assayed in a conventional 5-hour ^{51}Cr release assay. Target cells were autologous (HLA Al, A29; B8, B44) and allogeneic EBV-BLCL, either uninfected or infected at a multiplicity of infection of 10 plaque forming units per cell 16 hours previously with different recombinant vaccinia viruses. In some assays, synthetic peptides were added to the assay medium at different concentrations. Peptides were synthesized by the solid-phase method on an automated peptide synthesizer with FMOC chemistry, purified, and their identity confirmed. CTL assay results are expressed as the % specific ^{51}Cr release, calculated as 100 x (experimental release-spontaneous release)/(maximum release-spontaneous release).

Limiting dilution analysis of specific CTLp frequency. Precursor frequencies of specific CTL were estimated by limiting dilution analysis. Cryopreserved patient PBMC were plated at a range of dilutions and were restimulated by in vitro culture in IL-2-containing medium with an anti-CD3 antibody and irradiated allogeneic PBMC from a normal donor to allow expansion of CTL. Wells were then split and assayed for cytotoxicity on ^{51}Cr-labeled autologous target cells infected with different recombinant vaccinia viruses or coated with synthetic peptide AENLWVTVY. The fraction of non-responding wells was calculated by determining the number of wells in which ^{51}Cr release did not exceed 10% specific lysis. Precursor frequencies were then estimated by single hit model Poisson distribution analysis.

Statistical Analysis. Descriptive statistics as well as nonparametric analyses were used to evaluate the data (55). Spearman rank correlations and Pearson linear correlations were performed. Linear and non-linear regression analyses were employed to define quantitative relationships between variables. The Chi square test was used to compare detection frequencies of various virologic assay measurements in relation to patient groups exhibiting $CD4^+$ cell ranges of <200, 200-500, and >500/mm^3. The Kruskal-Wallis test was used to compare viral marker levels among the different groups, and the Wilcoxon signed rank test was used for one-sample or paired two-sample data analysis to assess the significance of treatment-related viral load changes.

Results

Branched DNA signal amplification of plasma viral RNA, quantitative plasma virus cultures, and regular p24 Ag assays were performed on plasma specimens from a total of 152 HIV-1 seropositive patients at the two study sites. Represented in this group of patients were 8 subjects with acute (primary) infection and 144 subjects with chronic infection. Eighty-six percent (131 of 152) of all patients, including those with acute infection, had detectable bDNA values above the 10,000 RNA Eq/ml assay sensitivity cutoff. bDNA values for plasma viral RNA ranged from $<10^4$ Eq/ml to 9×10^7 Eq/ml. Fifty-eight percent (88 of 152) of patients had culturable plasma virus and 46% (70 of 152) had measurable p24 Ag. QC-PCR analysis of plasma viral RNA and ICD-p24 Ag testing were performed on specimens from the 72 patients at the UAB site (6 with acute infection and 66 with chronic infection). The sensitivities of QC-PCR and ICD-p24 Ag assays were 100% (72 of 72) and 61% (44 of 72), respectively. QC-PCR determined RNA values ranged from 10^2 to 2×10^7 molecules/ml. None of 119 HIV seronegative control subjects had positive marker results by any of the assays used.

Table 1 shows the relative sensitivities of the bDNA assay compared with the other assay methods as a function of $CD4^+$ lymphocyte counts in patients with chronic infection. For this analysis, the 8 subjects with acute illness were excluded since such patients are uniformly viremic and have viral load measurements that bear no consistent relationship to CD4 counts (8, 12, 13). Eighty-five percent of patients had viral RNA detectable by bDNA compared with 100% by QC-PCR and 56% by viral culture. Forty-three percent of subjects had detectable p24 Ag and this was increased to 58% by use of the ICD-p24 Ag assay. Except for QC-PCR, which was positive in all subjects tested, there was an obvious, highly significant inverse correlation between CD4 counts and the frequency of detection of each virologic marker ($p < 0.001$ for all). The mean bDNA values for plasma viral RNA in patients with $CD4^+$ cell counts of $<200/mm^3$, $200\text{-}500/mm^3$, and $>500/mm^3$ were 583×10^3, 71×10^3, and 45×10^3 Eq/ml, respectively. bDNA values for patients with $CD4^+$ cells $<200/mm^3$ were significantly greater than for patients with higher CD4 counts ($p<0.0001$). Of the 123 bDNA positive patients with chronic infection (Table 1), one had a value between 10^7 and 10^8 RNA

Eq/ml, five had values between 10^6 and 10^7, 56 had values between 10^5 and 10^6, 61 had values between 10^4 and 10^5 (including 13 patients with bDNA values less than 25,000 RNA Eq/ml). There were no statistically significant differences in detection rates for any of the viral markers between the two study sites except for plasma virus cultures which were more commonly positive at ADARC (65%) than at UAB (44%) ($p < 0.05$).

The availability of replicate plasma samples from the UAB study site for which bDNA, QC-PCR, p24 Ag, ICD-p24 Ag, and quantitative viral culture results were all determined enabled us to analyze directly the quantitative relationship between bDNA values and these other viral markers. Figure 1 depicts these data, showing positive correlations between bDNA and QC-PCR, bDNA and ICD-p24 Ag, and bDNA and culture results. Table 2 summarizes the Spearman rank correlation coefficients among these viral load markers and between each of them and CD4 levels. bDNA and QC-PCR correlated most strongly with $CD4^+$ cells ($r=-0.72$ and -0.75, $p < 0.0001$ for both). Among the viral load markers, bDNA and QC-PCR correlated with each other most strongly ($r=0.89$, $p<0.0001$). Pearson correlation analysis, which evaluates linear relationships, revealed a similarly strong correlation between $\log_{10}$ bDNA and $\log_{10}$ QC-PCR ($r=0.89$, $p<0.0001$). In order to evaluate further the quantitative relationship between plasma viral RNA levels determined by bDNA and those determined by QC-PCR, a linear regression analysis was performed of the data shown in Figure 1A. The best fit line is described by the equation log bDNA=0.93+0.80 * log QC-PCR ($R^2=0.81$, $p<0.0001$).

Inter-assay reproducibility of the bDNA assay was determined by quantifying one positive specimen and one negative specimen, in duplicate, over the course of 17 assay runs at ADARC and UAB. The overall (inter-laboratory and inter-assay) mean and standard deviation for quantitation of the positive specimen were 66.6×10^3 and 13.7×10^3 RNA Eq/ml, respectively, resulting in a 21% coefficient of variation. In all of the 17 assay runs, the negative control quantitated below the assay cutoff of 10^4 RNA Eq/ml.

Having characterized in this patient population the bDNA assay's sensitivity (85%), specificity (100%), reproducibility (coefficient of variation 21%), quantitative range (10^4-10^8 RNA Eq/ml), and

correlation with $CD4^+$ cell counts ($r = -0.72$, $p<0.0001$) and four direct virologic markers ($r=0.51$ to 0.89, $p<0.0001$ for all), we next sought to evaluate the assay's dynamic response in the clinical setting of primary (acute) HIV-1 infection and following the institution of antiretroviral therapy. Acute HIV-1 infection is characterized by high levels of viral replication with infectious virus titers, virion-associated RNA (determined by PCR methods), and plasma p24 Ag all reported to be high prior to antibody seroconversion (8, 12, 13). We thus determined plasma viral RNA levels by the bDNA method, along with infectious virus titers and p24 Ag levels, through the period of acute infection and seroconversion in two ADARC patients (Figure 2). Peak levels of plasma virion-associated RNA ranged from 4×10^6 to 9×10^7 Eq/ml and fell by 100-to 10,000 fold following antibody seroconversion. Infectious virus titers and p24 Ag levels paralleled those of bDNA. Plasma virus assayed by the bDNA method, but not by culture or p24 Ag assay, remained detectable throughout the 1-2 years of clinical follow-up. bDNA assays were also performed on plasma specimens from 6 patients at UAB who had acute HIV-1 infection. These determinations were made on single plasma specimens corresponding to time points of peak viremia based on QC-PCR and p24 Ag measurements (8). bDNA values for these patients ranged from 7×10^5 to 2×10^7 RNA Eq/ml while QC-PCR measurements of replicates of the same plasma specimens ranged from 4×10^5 to 2×10^7 RNA molecules/ml. RNA results determined by the two assay methods for these six patients were highly correlated ($r=0.87$, $p < 0.03$).

Finally, sequential plasma specimens from 12 patients beginning zidovudine therapy were analyzed by bDNA, QC-PCR, p24 Ag, and ICD-p24 Ag assays before, during, and one week after a 6 week course of drug therapy (Figure 3). Of note, all four assays were performed on batched samples so as to minimize inter-assay variability. Figure 3A shows that initiation of zidovudine therapy resulted in an immediate and generally sustained fall in viral RNA as measured by the bDNA assay. Discontinuation of zidovudine after week 6 led to an immediate increase and return to baseline of viral RNA. The viral RNA response to zidovudine therapy measured by bDNA and QC-PCR were virtually identical in kinetics and magnitude

(figure 3b), resulting in a statistically significant drop of both markers at week one to 27-32% of their baseline values ($p < 0.001$); changes in p24 Ag and ICD-p24 Ag paralleled those for viral RNA although they were of lesser magnitude, declining at week one to 55% of pretreatment levels. After discontinuing zidovudine at week six, viral RNA levels returned promptly to baseline, a change paralleled to a lesser degree by p24 Ag and ICD-p24 Ag. These results thus addressed objectives relevant to aim #1.

Studies were also conducted to address aims #2 and #3. Despite the obvious importance of viral replication in HIV-1 disease, relatively little quantitative information has been available regarding the kinetics of virus production and clearance *in vivo*, the rapidity of virus and $CD4^+$ cell population turnover, and the fixation rates of biologically-relevant viral mutations. This circumstance is largely due to the fact that previously available antiretroviral agents lacked sufficient potency to abrogate HIV-1 replication and methods to quantify virus and determine its genetic complexity were not sufficiently sensitive and accurate. We overcame these obstacles by treating subjects with new investigational agents that potently inhibit the HIV-1 reverse transcriptase (Nevirapine, NVP) and protease (ABT-538; L-735,524); by measuring viral load changes using sensitive new quantitative assays for plasma virus RNA; and by quantifying changes in viral genotype and phenotype in uncultured plasma and PBMCs using automated DNA sequencing and an *in situ* assay of RT function (54).

Twenty-two HIV-1 infected subjects with $CD4^+$ lymphocyte counts between 18 and 251/mm^3 (mean $\pm$ 1 S.D. = 102 $\pm$ 75 cells/mm^3) were treated with ABT-538 (n=10), L-735, 524 (n=8), or NVP (n=4) as part of phase I/IIA clinical studies. Plasma viral RNA levels in the 22 subjects at baseline ranged from $10^{4.6}$ to $10^{7.2}$ molecules/ml (geometric mean of $10^{5.5}$) and exhibited maximum declines generally within 2 to 4 weeks of initiating drug therapy (Figs. 4 and 5A). For ABT-538 and L-735,524 treated patients, virus titers fell by as much as $10^{3.9}$ fold (mean decrease of $10^{1.9}$ fold) whereas for NVP-treated patients virus fell by as much as $10^{2.0}$ fold (mean decrease of $10^{1.6}$ fold). The overall kinetics of virus decline during the initial weeks of therapy with all three agents corresponded to an exponential decay process (Fig. 4 and 5A).

The antiretroviral agents used in this study, despite their differing mechanisms of action, have a similar overall biological effect in that they block *de novo* infection of cells. Thus, the rate of elimination of plasma virus that we measured following the initiation of therapy is actually determined by two factors: the clearance rate of plasma virus *per se* and the elimination (or suppression) rate of pre-existing, virus-producing cells. To a good approximation, we can assume that virus-producing cells decline exponentially according to $y(t) = y(O)e^{\alpha t}$, where $y(t)$ denotes the concentration of virus-producing cells at time t after the initiation of treatment and α is the rate constant for the exponential decline. Similarly, we assume that free virus $v(t)$ is generated by virus-producing cells at the rate $ky(t)$ and declines exponentially with rate constant u. Thus, for the overall decline of free virus, we obtain $v(t) = v(O)[ue^{\alpha t} - ae^{ut}]/(u-\alpha)$. The kinetics are largely determined by the slower of the two decay processes. Since we have data only for the decline of free virus, and not for virus-producing cells, we cannot determine which of the two decay processes is rate-limiting. However, the half-life ($T_{½}$) of *neither* process can exceed that of the two combined. With these considerations in mind, we estimated the elimination rate of plasma virus and of virus-producing cells by three different methods: (i) first-order kinetic analysis of that segment of the viral elimination curve corresponding to the most rapid decline in plasma virus, generally somewhere between days 3 and 14; (ii) fitting of a simple exponential decay curve to all viral RNA determinations between day 0 and the nadir or inflection point (Fig. 4); and (iii) fitting of a compound decay curve that takes into account the two separate processes of elimination of free virus and virus-producing cells, as described above. Method (i) gives a T½ of 1.8 ± 0.9 days; method (ii) gives a $T_{½}$ of 3.0 ± 1.7 days; and method (iii) gives a $T_{½}$ of 2.0 ± 0.9 days for the slower of the two decay processes and a very similar value, 1.5 ± 0.5 days, for the faster one. These are averages (± 1 S.D.) for all 22 patients. Method (iii) arguably provides the most complete assessment of the data, whereas method (ii) provides a simpler interpretation (but slightly slower estimate) for virus decline because it fails to distinguish the initial delay in onset of antiviral activity due to the drug accumulation phase, and the time required for very recently infected cells to initiate virus expression, from the subsequent

phase of exponential virus decline. There were no significant differences in the viral clearance rates in subjects treated with ABT-538, L-735,524, or NVP, and there was also no correlation between the rate of virus clearance from plasma and either baseline CD4+ lymphocyte count or baseline viral RNA level.

As an independent approach for determining virus turnover and clearance of infected cells, we quantified serial changes in viral genotype and phenotype with respect to drug resistance in the plasma and PBMCs of four subjects treated with NVP (Fig. 5). NVP potently inhibits HIV-1 replication but selects for one or more codon substitutions in the RT gene. These mutations result in dramatic decreases (up to 1000-fold) in drug susceptibility and are associated with a corresponding loss of viral suppression *in vivo*. Genetic changes resulting in NVP-resistance can thus serve as a quantifiable molecular marker of virus turnover. A rapid decline in plasma viral RNA was observed following the institution of NVP therapy and this was associated with a reciprocal increase in $CD4^+$ lymphocyte counts (Figs. 5A and B). Both responses were of limited duration, returning to baseline within 6-20 weeks in these four patients. The proportion of virus in uncultured plasma and PBMCs that contained NVP resistance-conferring mutations (Fig. 5C) was determined by direct automated nucleotide sequencing of viral nucleic acid (Fig. 6). We first validated this method by reconstitution experiments, confirming its sensitivity for detecting RT mutants that comprise as little as 10% of the overall virus population. Defined mixtures of wildtype and mutant HIV-1 RT cDNA clones (differing only at the second base position of codon 190) were amplified and sequenced (Fig. 6A). Varying proportions of wildtype and mutant viral sequences present in the original DNA mixtures (mutant composition: 0%, 10%, 25%, 50%, 75%, and 100%) were faithfully represented in the relative peak-on-peak heights (and in the relative peak-on-peak areas) of cytosine (C) and guanine (G) residues at the second base position within this codon. Ratios of (mutant+wildtype) nucleotide peak heights expressed in arbitrary fluorescence units were as follows (predicted/observed): 0%/<10%; 10%/18%; 25%/29%; 50%/49%; 75%71% and 100%/94%.

We next determined the ability of direct population sequencing to quantify wild-type and mutant

viral RNA genomes in clinical specimens. Fig. 6B shows the sequence chromatograms of RT codons 179191 from virions pelleted directly from uncultured plasma specimens of subject 1625 before (day -7) and after (days +28 and +140) the initiation of NVP therapy. At day -7, all codons within the amino terminal half of the RT gene (codons 1-250), including those shown, were wild-type at positions associated with NVP resistance. However, after only 28 days of NVP therapy, the wild-type plasma virus population was completely replaced by a NVP-resistant mutant population differing from the wild-type virus at codon 190 (glycine to serine substitution). After 140 days of drug therapy, this codon had evolved further such that the plasma virus population consisted of an equal mixture of two drug-resistant strains, one containing G19OS and the other containing G19OA. There were no other NVP resistance-coffering mutations detectable within the viral RT gene. In all four subjects evaluated by direct viral population sequencing (Fig. 7), specific NVP resistance-conferring mutations within the RT gene could be unambiguously identified and subsequently confirmed by molecular cloning, expression, and drug susceptibility testing. In 11 cases, mutant virus increased rapidly in the plasma and virtually replaced wild-type virus after only 24 weeks of NVP therapy (Fig. 5C). By analyzing the rate of accumulation of resistant mutants in the plasma population, we could obtain an independent estimate of the turnover rate of free virus. The rise of drug-resistant mutant virus is influenced substantially by the preceding increase in the $CD4^+$ cell population (which provides additional resources for virus production) and therefore follows complex dynamics. However, we could obtain an estimate of these dynamics by making simplifying assumptions. We assume that wildtype virus declines exponentially with a decay rate α and that the drug-resistant mutant increases exponentially with the rate β. Thus, the ratio of mutant to wild-type virus increases exponentially at the combined rate $\alpha + \beta$. Our genetic RNA (cDNA) data allow us to estimate this sum. Knowing α from our data on virus decline, we get β 0.27, or a 32% daily virus production (average over 4 patients). Assuming that mutant virus rises exponentially, this corresponds to a doubling time of approximately 2 days which is in excellent agreement with the measured elimination half-life of 2.0 ± 0.9 days for plasma virus (Figs. 4 and 5A). Turnover of viral DNA

from wild-type to drug-resistant mutant in PBMCs was delayed and less complete compared to plasma virus, reaching levels of only 50-80% of the total PBMC-associated viral DNA population by week 20 (Fig. 5C). Measurement of the time required for resistant virus to spread in the PBMC population allowed us also to estimate the half-life of infected PBMCs. After complete turnover of mutant virus in the plasma pool, we may assume that PBMCs infected with wild-type virus decline exponentially at a rate *d*, whereas cells infected by mutant virus are generated at a constant rate, but also decline exponentially at rate *d*. With these simplifying assumptions, the rate at which the frequency of resistant virus in the PBMC population increases provides an estimate for the parameter *d* and hence for the half-life of infected PBMCs. We obtained a half-life of approximately 50-100 days. This means that the average half-life of infected PBMCs is very long and of the same order of magnitude as the half-life of uninfected PBMCs. Based on the long half-life of PBMCs, and the fact that these cells harbor predominantly wild-type virus at a time (days 14-28) when most virus in plasma is mutant we conclude that most PBMCs contribute comparatively little to plasma virus load. Instead, other cell populations, most likely in the lymphoreticular system, must be the major source of virus production.

Direct sequence analysis of viral nucleic acid revealed not only rapid initial turnover in viral populations but also continuing viral evolution with respect to drug resistance mutations. In subject 1625 (Figure 3, top panel), wild-type virus in plasma was completely replaced after 28 days of NVP therapy by mutant virus (G19OS) which in turn evolved by day 140 into a mixture of G190S and G190A in subject 1624 (Figure 7, middle panel), two codon changes conferring NVP-resistance occurred. A G190A substitution appeared in plasma virus at day 14 and a Y181C appeared at day 42. Similarly, in-subject 1605 (not shown), a Y181C mutation appeared in plasma at day 14 and a Y188L mutation at day 28. The sequential changes in plasma virus were mirrored by similar changes in PBMCs at later timepoints. In subject 1619, the pattern of resistance changes was even more complex (Fig. 7, bottom panel). By day 14, approximately 70% of plasma virus contained a G190A mutation. By day +28, this mutant population was largely replaced by virus

containing a Y188F/L substitution. By day 84, still another major shift in the viral quasispecies occurred, this time resulting in a population of viruses containing mutations at both Y181C and G190A. Finally, by day 288 the viral population in plasma consisted exclusively of a mutant exhibiting a single tyrosine to isoleucine substitution at position 181 (Y181I); mutations at codons 188 and 190 were not present in this virus population. All of these amino acid substitutions at RT codons 181, 188, and 190 were shown in our own *in situ* expression studies and by others to confer high level NVP resistance. The direct sequence analyses thus demonstrate that major changes in the HIV-1 quasispecies occur quickly and continuously in response to selection pressures and that these changes are reflected first and most prominently in the plasma virus compartment.

Because direct sequence analysis of viral mixtures provides only semiquantitative information and does not distinguish between viruses with functional versus defective RT genes, we employed another method for quantifying virus turnover in uncultured plasma and PBMC compartments. Full-length RT genes were amplified by PCR, cloned into pLG18-1, expressed in *E. coli,* and tested individually for enzymatic function and NVP susceptibility by *in situ* assay. Table 3 shows the results of these analyses. For subject 1625 at day 7, 100% (80/80) of RT clones from plasma and 100% (163/163) of RT clones from PBMCs expressed enzyme that was sensitive to NVP inhibition. By day 14, however, 62% of plasma-derived clones expressed enzyme that was resistant to NVP, and by days 28, 84, and 140, 100% were resistant. Conversely, at day 14, 0% of PBMC-derived clones expressed NVP-resistant enzyme, and even after 28, 84, and 140 days, only 48-75% of clones were resistant. Similar results were obtained for the other study subjects (see Table 3). Thus, the kinetics of virus population turnover determined by a quantitative RT *in situ* expression assay corresponded closely with those determined by direct population sequencing (Figures 5C).

Plasma and PBMCs are known to harbor substantial proportions of defective or otherwise noninfectious virus. To determine if the viral genomes represented in total viral nucleic acid corresponded to infectious virus with respect to NVP resistance-conferring mutations, we co- cultivated PBMCs from three

of the study subjects (1605, 1624, 1625) with normal donor lymphoblasts in order to establish primary virus isolates. The RT genes of these cultured viruses, obtained before and after therapy, were cloned and sequenced in their entirety. RT codons associated with NVP susceptibility were completely concordant in cultured and uncultured virus strains. Furthermore, the virus isolates exhibited NVP susceptibility profiles consistent with their genotypes.

HIV-1 load *in vivo* is comprised of cell-free virus as well as substantial numbers of replication-active, latent, or defective viral genomes, all of which likely play a role in disease pathogenesis. The studies described above emphasized the dynamics of virus turnover in plasma and the clinical benefits associated with decreases in plasma viremia following antiretroviral therapy. Yet, antiretroviral regimens which maximally impact all viral "compartments" are likely to result in the greatest therapeutic gains and the longest delay in the development of drug resistance. It is thus essential to elucidate the numbers and half-lives of cell populations harboring active, latent, or defective viral forms. We utilized a combination of biologic (phenotypic) and genetic approaches to quantify these viral forms in peripheral blood mononuclear cells (PBMCs). Three subjects ($CD4^+$ cells 20-200/mm^3) were studied before and 14, 28, 42, and 140 days after initiation of Nevirapine (NVP) antiretroviral therapy. At baseline, the total viral DNA content of PBMCs determined by QC-PCR ranged from 450-800 molecules per 10^6 cells. Infectivity titers determined by biological cloning ranged from 17-29 infectious units per 10^6 total cells. After initiation of NVP therapy, infectious PBMC-associated virus titers fell significantly more (30-fold) than did the total number of HIV-1 DNA positive cells (2-fold). Clearance rates of specific virally-infected cell populations were determined by quantifying the elimination of wild-type virus and its replacement by drug-resistant mutant virus. The half-life of cells infected with total virus (defective plus competent) determined by direct population sequencing and *in situ* RT expression was approximately 50-100 days. The half-life of cells harboring replication-competent virus determined by biological cloning and phenotypic resistance testing was biphasic, reflecting separate cell populations containing either transcriptionally-active or latent virus infection:

approximately 90% of PBMCs containing replication-competent virus were eliminated within 14 days (T½ < 5 days), whereas another 1-10% of such cells persisted for at least 42 days. The results of these studies thus define three populations of virally-infected PBMCs. The largest population (approximately 450-800 cells per million) contains largely defective virus and exhibits a half-life of 50-100 days; a second population, much smaller (17-29 cells per million), contains activity replicating virus and is eliminated with a half-life of <5 days, similar to lymphoreticular cells responsible for sustaining plasma; a third population, still smaller (approximately 1 cell per 3.3 million), contains latent virus and exhibits a lifespan of at least 42 days. These findings indicate that although PBMCs harbor a predominately archival record of prior (abortive) viral infection, they also contain replication-competent genomes in both transcriptionally-active and latent states. These results provide a scientific framework with which to extend studies of viral and cellular dynamics into the lymphoreticular tissue compartments.

Changes in $CD4^+$ lymphocyte counts during the first 28 days of therapy could be assessed in 17 of our patients (Fig. 5B). $CD4^+$ cell members increased in every patient by between 41 and 830 cells/mm^3. For the entire group, the average increase was 186 ± 199 cells/mm^3 (mean ± 1 S.D.), or 268 ± 319% from baseline. Since $CD4^+$ lymphocytes increase in numbers because of (i) exponential proliferation of $CD4^+$ cells in peripheral tissue compartments, and/or (ii) constant (linear) production of $CD4^+$ cells from a pool of precursors, we analyzed our data based on each of these assumptions. The average percentage increase in cell number per day (assumption i) was 5.0 ± 3.1% (mean ± 1 S.D.). The average absolute increase in cell number per day (assumption ii) was 8.0 ± 7.8 cells/mm^3/day. Given that peripheral blood contains only 1/50th of the total body lymphocytes and that the average total blood volume is approximately 5 liters ($5x10^6$ mm^3), an increase of 8 cells/mm^3/day implies an overall steady-state $CD4^+$ cell turnover rate (where increases equal losses) of (50) x ($5x10^6$ mm^3) x (8 cells/mm^3/day), or 2 x 10^9 $CD4^+$ cells produced and destroyed each day.

The final set of experiments performed were undertaken to determine if viral specific CTL responses

might be sufficiently active against HIV-1 infected cells that turnover in the virus (and in infected cell populations) would occur in a manner similar to the effects of antiretroviral therapy. In many virus infections, including HIV-1 (56, 57), the virus-specific CD8 cytotoxic T lymphocyte (CTL) response plays a key role in viral clearance. Likewise, recent studies in animal models suggest that vaccine-induced protection against simian and feline immunodeficiency viruses is correlated with the induction of strong antigen-specific CTL responses (58, 59). HIV-1 -specific CTL activity has also been demonstrated in exposed seronegative individuals (60, 61), although the relative contributions made by the cell-mediated immune response and genetically-conferred resistance (62) to the lack of infection in these individuals remains unclear. Further suggestive evidence that virus-specific $CD8^+$ CTL make an important contribution to containment of virus replication during infection with HIV-1 comes from the observations that HIV-1-infected long-term nonprogressors have high levels of HIV-1-specific CTL precursors (CTLp) (63, 64), while HIV-1 -specific CTLp are lost during progression to AIDS (63-66). However the immunosuppressive nature of HIV-1-associated disease makes it difficult to assign cause and effect when a loss of immune function is observed in association with increasing viral titres in the end stages of the infection.

Study of the events taking place early after infection with HIV-1 has shown that virus-specific $CD8^+$ CTL responses are induced prior to seroconversion, and are temporally associated with the fall in viremia during acute infection (56, 57). Whilst these observations are consistent with the antiviral CTL response playing a role in containment of virus replication early after infection, it has been suggested that the decline in primary viremia may simply be a result of population dynamics, with virus replication decreasing due to a diminishing pool of activated lymphocytes (67). To provide definitive evidence that the early CTL response does have an impact on virus replication *in vivo,* we performed an in-depth analysis of CTL-virus interactions at early times post-infection in a patient (WEAU; subject #1 in Clark *et al.* Ref. 12) who presented with acute symptomatic HIV-1 infection. This particular patient was chosen for study because preliminary analysis (57, 68) suggested that his early CTL response may have been of limited epitope

specificity. In other virus infections, antiviral CTL reach very high frequencies at early times post-infection (69). We reasoned that if the antiviral CTL response is indeed an important controlling force restricting HIV-1 replication during the acute phase of the infection, then in a patient where the early HIV-1 specific CTL response was directed against a small number of viral epitopes, selection for HIV-1 variants bearing mutations conferring escape from CTL recognition might be observed (70), analogous to viral mutations leading to antiretroviral drug escape (54).

Patient WEAU was a homosexual male who presented with symptomatic primary HIV-1 infection 20 days following a single sexual encounter with a patient with AIDS, shown by viral DNA sequence analysis to represent the source of infection (12). Infectious virus was present in the plasma of WEAU at the earliest timepoint tested [15 days following the onset of symptoms (DFOSx)], but the titre declined rapidly, reaching undetectable levels by 23 DFOSx when seroconversion occurred (Table 4). Plasma virion-associated RNA and viral p24 antigen each peaked between 20 and 23 DFOSx. Plasma viral RNA reached its nadir at day 72, and thereafter increased coincident with a decline in CD4 lymphocyte count. Initial analysis of the CTL response mounted by this patient revealed that HIV-1-specific, MHC-restricted $CD8^+$ CTL activity could be detected at the earliest timepoint available for study, 16 DFOSx, and that this early CTL response was directed predominantly against the viral envelope glycoprotein, gp160 (57).

To allow a more detailed characterization of the specificity of the primary CTL response, recombinant vaccinia viruses were constructed which expressed full-length gp160 derived from the autologous HIV-1 strain in patient WEAU 15 DFOSx, as well as serial N- and C- terminally truncated sections of this protein. As shown in Figure 8, polyclonal CTL cryopreserved from patient WEAU 20 DFOSx mediated MHC-restricted lysis of target cells expressing the full-length autologous virus gp160 but not other HIV-1 proteins (gag, pol, tat or nef). Within the autologous gp160 protein, all the epitope(s) recognized were located within the N-terminal 110 aa (Figure 8). When a panel of ten gp160-specific MHC-restricted CTL lines/clones derived from patient WEAU 20 DFOSx were screened, they were also all found

to recognize only epitope(s) within aa 1-110 (not shown). To determine the location of these epitope(s), overlapping synthetic peptides corresponding to the N-terminal 110 aa of the day 15 autologous virus gp160 sequence from patient WEAU were used. As representative results in Figure 9A illustrate, all clones tested recognized a single peptide corresponding to gp160 aa 25-41. Using a panel of target cells known to have one or more HLA-A or B alleles in common with patient WEAU (whose HLA type is A1, A29; B8, B44), the restricting HLA molecule for these clones was shown to be HLA-B44 (Figure 9B). A peptide binding motif has been defined for HLA-B44: the anchor residues are E at position 2 and Y or F at position 9, with a second Y or F residue at position 10 forming an auxiliary anchor (71). A sequence corresponding to this motif was contained in gp160 aa 25-41, at aa 30-39. Peptides of differing lengths based on the gp160 aa 29-39 sequence of patient WEAU's day 15 autologous virus were tested for their relative ability to sensitize autologous target cells for lysis by day 20 WEAU CTL. Both polyclonal CTL and CTL clones were able to recognize a peptide corresponding to gp160 aa 29-39 (Figure 9c and data not shown), confirming that as predicted, the epitope was contained in this sequence. Different CTL clones were found to prefer either aa 30-38 or aa 30-39 as the optimal length of epitope (examples are shown in Figure 9c). Polyclonal CTL from patient WEAU 23 DFOSx recognized both peptides efficiently, giving 50% maximal lysis of target cells presenting each peptide at $5x10^{-6}$ - 10^{-7}M.

Limiting dilution analysis indicated that the frequency of CTLp recognizing the gp160 aa 30-38 epitope in recombinant vaccinia virus vM1 was approximately 1 per 17 PBMC 16 DFOSx (not shown). By contrast, the frequency of CTLp recognizing epitopes elsewhere in gp160 (recombinant vaccinia virus vM9) was below the limit of detection in this assay (< 1 per 2000 PBMC), as was the frequency of CTLp directed against vSC8, the control vaccinia virus. All detectable HIV-1-specific CTL activity mediated by PBMC cryopreserved from patient WEAU 16 DFOSx was thus directed against a strongly immunodominant viral epitope, gp160 aa 30-38(9). From the very high frequency 16 DFOSx, the frequency of CTLp directed against this epitope subsequently declined to 1 per 49 PBMC 23 DFOSx, and 1 per 7400 PBMC by 136

DFOSx.

To determine whether the early CTL response mounted by patient WEAU exerted a biologically significant selective pressure on the virus population, we performed a quantitative analysis of sequence changes occurring throughout gp160 over time. Because circulating plasma virus most accurately represents the actively replicating virus population *in vivo,* (54, 72) we sequenced and compared serial specimens of *uncultured* plasma virus RNA (cDNA). At the initial timepoint (16 DFOSx), plasma virus was essentially homogeneous as assessed by direct population sequencing of virion RNA (cDNA) (example shown in Figure 10a) and by sequence analysis of individually cloned envelope genes (Figure 10b). In other studies (not shown), *gag, vpu, env,* and *nef* genes were amplified by PCR from *uncultured* PBMC DNA of this patient (10-20 sequences per gene) and found to be similarly homogeneous (<0.5% variation) at this timepoint. Analysis of the *env* gene sequences from plasma virus of patient WEAU between 16 and 136 DFOSx revealed progressive replacement of the initial virus population by one differing primarily at aa 31 (Figure 10). Figure 10A shows that this turnover in the plasma virus population was rapid and complete by day 136. Figure 10B demonstrates further evidence for the strong selective forces acting on this CTL epitope (aa 30-38) since changes within the epitope were numerous and accumulative whereas changes outside it were less frequent and sporadic. For example, within the CTL epitope, of the 48 *env* clones shown in Figure 10B (boxed area), there were a total of 432 (9aa x 48 clones) potential sites for aa substitution: 37 (8.6%) were changed. In the immediately adjacent sequences shown, there were 2688 (56 aa x 48 clones) potential sites for aa substitution but only 11 (0.4%) changes. Thus, changes within the CTL epitope exceeded by 21-fold those in surrounding areas of *env.* Moreover, the proportion of nonsynonymous nucleotide changes within the CTL epitope (37/38; 97.4%) exceeded that in the surrounding *env* regions (11/16; 68.8%) by 1.4-fold. To further evaluate if the frequency and rapidity of sequence changes observed at positions 30-38 were unique, direct viral RNA (cDNA) population sequencing of the amino-terminal half of gp160 (430 aa) was performed on plasma specimens corresponding to days 16, 30, 44, and 72 DFOSx; the *only* changes observed

were those at position 31 (E to G/A). To complete the analysis of HIV-1 quasispecies evolution in this patient, population sequencing of the full-length gp160 (860 aa) from plasma viral RNA (cDNA) from day 136 DFOSx, together with sequence analysis of three full-length gp160 molecular clones derived from the same viral RNA (cDNA) were compared with the viral sequences from day 16. Here, for the first time, changes in addition to those occurring at codon 31 were identified. These included substitutions at aa 356 (K to E), 400 (H to Y), 402 (N to D), 407 (N to D), 815 (V to I), 819 (N to D), and 857 (A to T).

To determine whether the observed changes in the gp160 aa 30-38 epitopic sequence affected CTL recognition of this epitope, synthetic peptides corresponding to aa 30-38 of the predominant mutant virus gp160 sequences were tested for their ability to sensitize autologous target cells for lysis by polyclonal CTL and CTL clones derived from patient WEAU 16-20 DFOSx. Unlike the wild-type peptide, peptides corresponding to the aa 31 mutant virus sequences which first emerged 30-72 DFOSx and became predominant by 136 DFOSx were not recognized by polyclonal CTL cryopreserved from patient WEAU 16 DFOSx (not shown), or by CTL clones derived from the patient 20 DFOSx (Figure 11). This could either be due to failure of these mutant peptides to bind to HLA-B44, or to lack of recognition of the peptides by CTL following MHC binding: the former seems more likely given that the gp160 aa 31 E residue forms one of the dominant MHC anchor residues in the HLA-B44 peptide binding motif (71). Interestingly, virus variants with an aa substitution from A to T at position 30 were transiently observed at 44 and 72 DFOSx but were not selected for by 136 DFOSx, and the corresponding peptide was efficiently recognized by epitope-specific CTL (Figure 11). Thus, unlike the early (day 16) or transient (A30T; day 44) viral population, mutants at position 31 (E31G; E31A) were able to escape recognition by the primary CTL response mounted by this patient and accumulated progressively through day 136.

The emergence of a mutant virus population able to escape recognition by the primary HIV-l-specific CTL response in patient WEAU was not accompanied by a dramatic increase in the plasma viral load in this patient (Table 4). Analysis of the CTL activity mediated by PBMC cryopreserved from patient

WEAU 30 DFOSx (Figure 12) revealed that even by this relatively early timepoint responses were detectable to several new viral epitopes, including at least one in gp160 aa 111-860, plus epitopes in gag, pol and nef. CTL of these novel specificities would be capable of recognizing their antigens associated with the gp160 aa 31 mutant virus population, and thus could account for its continued containment.

Discussion

A number of investigative groups have independently developed and reported novel assay methods for quantifying HIV-1 RNA in plasma (8, 9, 23, 38, 40, 44, 45, 47-51). In the early stages of these assays' development, discordant results were frequently reported regarding the frequency of detection, magnitude, and stability of HIV-1 RNA in plasma (8, 9, 23, 24, 38, 40-51). More recently, primarily because of technical improvements, consistent and reproducible findings linking plasma viral RNA to HIV-1 pathogenesis and natural history have been reported (8, 9, 23, 38, 56). Using an internally controlled quantitative competitive RT-PCR method (QC-PCR), Piatak et al. (8) have demonstrated conclusively that: (i) virion-associated HIV-1 RNA can be detected in virtually all seropositive individuals regardless of disease stage; (ii) viral RNA levels generally range from 10^2 to 10^7 molecules per milliliter of plasma, (iii) viral RNA levels correlate significantly with other virologic load markers such as infectious plasma virus and p24 Ag, with advanced clinical stages, and with low $CD4^+$ cell counts; and (iv) viral RNA levels fall significantly in association with seroconversion and following the institution of antiretroviral therapy.

Using a different RT-PCR assay procedure, Winters *et al.* (9) reported findings similar to those of Piatak, detecting HIV-1 RNA levels in the range of 10^2 to 10^6 molecules per ml in >95% of infected subjects. Furthermore, these investigators performed rigorous analyses of the natural biological variation of HIV-1 RNA in plasma, of assay reproducibility, and of the stability of plasma virus with long-term storage. They found a median intraassay reproducibility of $\log_{10}$ 0.15 RNA molecules per ml, median interassay reproducibility of $\log_{10}$ 0.25 molecules per ml, biological variation in viral RNA levels of $\log_{10}$ 0.30 molecules per ml in patients on no therapy or unchanged therapy, and stability of virion-associated RNA in

plasma stored at -70°C for up to one year. Coombs, *et al.* (46), and Aoki-Sei *et al.* (38), using still other methods for quantifying plasma virus, also demonstrated consistent and reproducible virus quantitation in fresh and stored human plasma.

Based on these reports and others, there has been increasing interest and concentration on the part of clinical investigators, clinicians, and patients alike to explore the use of plasma viral load measurements to assess drug activity in clinical trials and in the setting of individual patient management. In this context, however, it is important to emphasize that only the more technically demanding research based assays described above have been evaluated clinically. While there is the expectation that commercial assays designed for use in clinical or clinical research laboratories will yield data similar to those of basic research assays, it is essential to evaluate this question formally. Lin *et al.* (39) have provided important data in this regard demonstrating that six different HIV-1 RNA assay methods, including two commercial tests (Chiron and Roche), were able to discriminate and accurately rank a constructed 10-fold dilution series of cultured HIV-1 virus spiked into normal human plasma. Furthermore, these assays could discriminate between 19 positive or negative clinical samples and between positive samples with high versus low virus loads, although method-specific differences in the quantitative results for individual patient specimens were as high as 100-fold. Nonetheless, the reproducibility of certain of the assays, including the Chiron bDNA assay, was such that an empirical fourfold difference in RNA levels could be viewed as significant and it was recommended that they be advanced to clinical trial evaluation (39).

The present study represents one of the first large clinical research evaluations of the Chiron branched DNA signal amplification assay and it is unique in having a combination of other viral load measurements, including QC-PCR determinations of viral RNA, available for direct comparison. In this study, we determined bDNA values in 152 patients at all stages of infection and in 119 HIV negative controls and we related this information to clinical stage, $CD4^+$ cell counts, results of other viral load measurements, clinical course following acute infection, and response to therapy. The sensitivity of the bDNA assay in this

relatively advanced patient population (83 patients with $CD4^+$ counts <200/mm^3, 37 with counts from 200-500/mm^3, and 24 with counts >500/mm^3) was 85%. Its specificity was 100%, reproducibility 21% (coefficient of variation), working range for clinical samples 10^4-10^8 RNA Eq/ml, correlation (r value) with $CD4^+$ counts -0.72 ($p<0.0001$), and correlation with four other viral markers ranging from 0.51 to 0.89 ($p<0.0001$ for all). Of note, the rank correlation coefficients (Table 2) were strongest between bDNA and QC-PCR (0.89), followed by bDNA and culturable virus (0.72), and then by bDNA and ICD-p24 Ag (0.56) and p24 Ag (0.51). In comparison, p24 Ag and ICD-p24 Ag were correlated at the 0.90 level. These empiric findings are consistent with expectations based on the viral components targeted by the respective assays: bDNA and QC-PCR detect total virion-associated RNA, plasma cultures detect infectious virus, and p24 Ag and ICD-p24 Ag detect the various forms of virion- and non-virion-associated core antigen.

Theoretically, results of the bDNA and QC-PCR assays should be very similar or identical since they both measure viral RNA from pelleted virus. In fact, results of the two assays were highly correlated (Spearman rank and Pearson correlation coefficients of 0.89, $p<0.0001$ for both) over a broad range of values from 10^4 to 2×10^7 viral RNA molecules/ml (Table 2 and Figure 1). In a total of 72 patients for whom bDNA and QC-PCR data were available (Table 1 plus six patients with primary infection), 75% had bDNA and QC-PCR results that differed by less than 0.5 $\log_{10}$; 99% of patients had bDNA and QC-PCR results that differed by less than 1.0 $\log_{10}$. Regression analyses revealed highly significant correlations between bDNA and QC-PCR results, indicating a nearly one to one relationship between bDNA and QC-PCR values over a 3 $\log_{10}$ range. Further analysis revealed a small but statistically significant method-associated trend for QC-PCR results to exceed bDNA results by an average of 0.168 $\log_{10}$ ($p < 0.001$). The fact that two independent viral RNA assays, based on completely different amplification strategies and having differently prepared quantitative standards, yielded nearly the same quantitative results for clinical samples over a 3 $\log_{10}$ dynamic range is important. Such data provide independent and mutual confirmation of the quantitative values for plasma viral RNA recorded by these assays. Other reports (24, 44) suggesting that plasma viral

RNA levels are generally 10 to 100 fold lower than those we determined by the bDNA and QC-PCR assays likely reflect the use of different assay methods, different quantitative standards, or less well preserved clinical specimens. The technically demanding nature of target (PCR) and signal (bDNA) amplification assays will require the use of common assay standards by laboratories performing these assays in clinical trial settings and planning to analyze data collectively.

Overall reproducibility of the bDNA results reported in this study was estimated by quantifying replicates of a single HIV-1 positive specimen and a single negative specimen over the course of 17 assay runs at ADARC and UAB. The overall inter-laboratory and inter-assay mean and standard deviation for the positive specimen was $66.6 \times 10^3 \pm 13.7 \times 10^3$ RNA Eq/ml, resulting in a coefficient of variation of 21%. In three other studies of bDNA assay reproducibility (39, 45, 57) in which as many as 12 different operators performed the test, overall coefficients of variation ranged from 18% to 23%. In the ACTG Virology Working Group study (39), assay reproducibility was determined by pooled standard deviations of results on pairs of blinded patient samples. In that study, the Chiron bDNA assay exhibited the smallest pooled standard deviation, and thus the greatest reproducibility, of all tests analyzed. Taken together, the results of the five studies indicate that differences in viral RNA results of as little as twofold, within or between assay runs, would be expected to be significant at the $p<0.05$ level.

An important question to investigators involved in many different types of clinical HIV-1 research (including antiretroviral chemotherapy, immunotherapy, natural history, pathogenesis and vaccine efforts) is which plasma viral RNA assays are most useful for quantifying virus load *in vivo*. In our view, a qualified answer is necessary depending on the patient population under study, baseline ranges in viral load, sensitivity and accuracy of measurements required to answer the questions posed, as well as feasibility issues such as commercial availability and ease of use of an assay method. The current study provides data for the Chiron bDNA assay relating to each of these issues. Adults at all clinical stages were evaluated, and even those with greater than 500 $CD4^+$ cells/mm^3 generally had detectable bDNA values, albeit at lower levels (45 ± 40 X

10^3 RNA Eq/ml, mean ± 1 S.D.). Enhancing the sensitivity of the bDNA assay from a lower cutoff limit of 10^4 RNA Eq/ml to 5 X 10^3 RNA Eq/ml, as has been done recently (unpublished data), will further increase the proportion of HIV-1 infected individuals with detectable viral RNA and will expand by twofold the range over which changes in viral load can be quantified. Sample volume requirements (duplicate 1 ml plasma specimens) specified for the bDNA assay can be problematic for pediatric studies or certain other applications; we successfully used sample volumes as low as 0.25 ml. Accuracy of the bDNA assay over a 3 $\log_{10}$ range (10^4 to 2 X 10^7 RNA Eq/ml) was independently validated by direct comparison of assay results with QC-PCR determined values using replicate plasma samples. Facility of use of the bDNA assay was confirmed by test performance at two clinical sites (ADARC and UAB).

Plasma viral RNA determinations by the Genelabs QC-PCR assay were employed for comparison with bDNA results in this study. Whereas the bDNA assay was able to detect and quantify HIV-1 RNA in the plasma of 62 of 72 UAB subjects (Table 1 plus six patients with acute infection), the QC-PCR assay was positive in all 72. The mean QC-PCR level in the ten subjects with negative (<10^4 RNA Eq/ml) bDNA results was $10^{4.10}$ RNA molecules/ml with a standard deviation of $\log_{10}$ 0.83. The accuracy of the QC-PCR assay has been determined experimentally by measuring recombinant HIV-1 RNA and DNA standards of known concentration, by quantifying viral RNA in culture supernatants for which virion particle counts were independently determined, and now by direct comparison with bDNA results on clinical samples. The precision and reproducibility of the QC-PCR has been determined to be approximately 20-25% (8). Because of the routine sensitivity of the QC-PCR assay of approximately 2,000 RNA molecules/ml, and its ability to detect and quantify plasma viral RNA at levels as low as 100 molecules per ml on an as needed basis, this assay has proven to be particularly useful in studies where accurate quantitation of viral RNA in the range of 100 to 50,000 molecules per ml is important.

A Roche Molecular Systems RT-PCR assay for quantifying plasma viral RNA has been described and its performance characteristics for detecting and quantifying HIV-1 RNA expressed from plasmid

vectors and in culture supernatants reported in detail (44). More limited data is available regarding the performance of this assay on clinical specimens (39, 44). If the sensitivity (200 HIV-1 RNA copies per ml plasma), low sample volume requirements (200 μl plasma), accuracy, and reproducibility reported for the research based assay (44) are maintained in a commercially available assay, then it will represent an important experimental tool for clinical AIDS research.

Previously, it was shown that lymphoreticular tissues serve as the primary reservoir and site of replication for HIV-1 and that virtually all HIV-1 infected individuals, regardless of clinical stage, exhibit persistent plasma viremia in the range of 10^2 to 10^7 virions per milliliter (8). However, the dynamic contributions of virus production and clearance, and of $CD4^+$ cell infection and turnover, to the clinical "steady-state" were obscure. The present report demonstrates by virus quantitation and mutation fixation rates that the composite lifespan of plasma virus and of virus-producing cells is remarkably short ($T_{1/2}$ = 2.0 $\pm$ 0.9 days). This holds true for patients with $CD4^+$ lymphocyte counts as low as 18 cells/mm^3 and as high as 355 cells/mm^3. Importantly, these findings were made in patients treated with three different antiretroviral agents having two entirely different mechanisms of action and using three different experimental approaches for assessing virus turnover. The viral kinetics observed thus cannot be explained by a unique or unforeseen drug effect or a peculiarity of any particular virologic assay method. Moreover, when new cycles of infection are interrupted by potent antiretroviral therapy, plasma virus levels fall abruptly by an average of 99%, and in some cases by as much as 99.99% (10,000-fold). This result indicates that the vast majority of circulating plasma virus derives from continuous rounds of *de novo* virus infection, replication, and cell turnover, and not from cells that produce virus chronically or are latently-infected and become activated.

The identity and location of this actively replicating cell population is not known but appears not to reside in the PBMC pool. Nevertheless, PBMCs traffic through secondary lymphoid organs and to some extent are in equilibrium with these cells. It is thus possible that a small fraction of PBMCs, like a small fraction of activated lymphoreticular cells, could contribute importantly to viremia.

The magnitude of ongoing virus infection and production required to sustain steady-state levels of viremia is extraordinary: Based on a virus $T_{1/2}$ of 2.0 days and first-order clearance kinetics ($v(t) = v(0)e^{-\alpha t}$, where $\alpha = 0.693/T_{1/2}$), 30% or more of the total virus population in plasma must be replenished *daily*. For a typical HIV-1 infected individual with a plasma virus titer equaling the pretreatment geometric mean in this study ($10^{5.5}$ RNA molecules per ml / 2 RNA molecules per virion = $10^{5.2}$ virions/ml) and a plasma volume of 3 liters, this amounts to (0.30) x ($10^{5.2}$) x ($3x10^3$) = 1.1 x 10^8 virions per day (range for all 22 subjects = 2 x 10^7 to 7 x 10^9). Even this may be a substantial underestimate of virus expression since virions may be inefficiently transported from the interstitial extravascular spaces into the plasma compartment and viral protein expression alone (short of mature particle formation) may result in cytopathy or immune-mediated destruction. Because the half-life of cells producing the majority of plasma virus cannot exceed 2.0 days, at least 30% of these cells must also be replaced *daily*. In our patients, we estimated the rate of $CD4^+$ lymphocyte turnover to be, on average, 2 x 10^9 cells per day, or about 5% of the total $CD4^+$ lymphocyte population depending on clinical stage. This rapid and ongoing recruitment of $CD4^+$ cells into a short-lived virus-expressing pool likely explains the abrupt increase in $CD4^+$ lymphocyte numbers that is observed immediately following the initiation of potent antiretroviral therapy and suggests the possibility of successful immunological reconstitution even in late-stage disease if effective control of viral replication can be sustained.

The kinetics of virus and $CD4^+$ lymphocyte production and clearance reported here have a number of biological and clinical implications. First, they are indicative of a dynamic process involving continuous rounds of *de novo* virus infection, replication, and rapid cell turnover that likely represents a primary driving force underlying HIV-1 pathogenesis. Second, the demonstration of rapid and virtually complete replacement of wild-type virus by drug-resistant virus in plasma after only 14-28 days of drug therapy is a striking example of the capacity of the virus for biologically-relevant change. In particular, this implies that HIV-1 must have enormous potential to evolve in response to selection pressures as exerted by the immune

system, which we have now demonstrated (figures 8-12). Although other studies have provided some evidence that virus turnover occurs sooner in plasma than in PBMCs, the data presented here shows this phenomenon most clearly. A similar experimental approach involving the genotypic and phenotypic analysis of plasma virus could be helpful in identifying viral mutations and selection pressures involved in resistance to other drugs, immune surveillance, and viral pathogenicity. Third, the difference in lifespan between virus-producing cells and latently-infected cells (PBMCs) suggests that virus expression *per se* is directly involved in $CD4^+$ cell destruction. The data do not suggest an "innocent bystander" mechanism of cell killing whereby uninfected or latently-infected cells are indirectly targeted for destruction by adsorption of viral proteins or by autoimmune reactivities.

Although we have emphasized that most virus in plasma derives from an actively-replicating short-lived population of cells, latently-infected cells that become activated or chronically-producing cells which generate proportionately less virus (and thus don't contribute substantially to the plasma virus pool) may nonetheless play an important role in HIV-1 pathogenesis. These cells far outnumber the actively-replicating pool and the diversity of their constituent viral genomes represents a potentially important source of clinically relevant variants, including those conferring drug resistance. In future studies, it will be important not only to discern the specific elimination rates of free virus and of the most actively producing cells but also the dynamics of virus replication and cell turnover in other cell populations and in patients at earlier stages of infection. Such information will be essential to developing a better understanding of HIV-1 pathogenesis and a more rational approach to therapeutic intervention.

In addition to viral dynamics following antiretroviral therapy, we also provide evidence here that the early HIV-1-specific CTL response exerts a substantial controlling pressure on virus replication *in vivo*. The present study is unique in that it represents a detailed molecular analysis of CTL-virus interactions very early after HIV-1 infection, prior to seroconversion and even before viral RNA had achieved peak titers. The first timepoint analyzed, 15 DFOSx, was exactly 35 days after the patient was infected by HIV-1 (12). The

kinetics of decline in plasma viremia coincided temporally with the appearance, first of a strong CTL response focused on a highly immunodominant epitope at gp160 aa 30-38, and shortly thereafter, of broader CTL reactivity. Evidence for a strong and biologically important selective pressure exerted by CTL was provided by the observation that by 30-44 DFOSx CTL escape mutants were detectable. By 136 DFOSx, there had been complete replacement of the transmitted virus strain (which had initially replicated to high titers) by a mutant population that differed primarily at aa 31. The cellular compartments harboring replicating virus early in infection were thus largely eliminated and replaced by cells infected with CTL escape variants by this timepoint.

The magnitude of wild-type virus decline, the kinetics of mutant virus appearance, and the genetic pathways by which virus escaped CTL recognition, bear certain similarities to viral dynamics in the setting of antiretroviral drug therapy. For example, between 16 and 72 DFOSx, wild-type virus in the plasma (defined by aa 30-38 sequence AENLWVTVY) declined from 200,000-300,000 RNA molecules per milliliter to approximately 1,000 (10% of 11,400). Mutant virus first appeared at 30 DFOSx and evolved in a complex pattern until a best-fit population not recognized by HLA B44-restricted CTL came to predominate at day 136. Viral evolution to the aa 30-38 escape variant would likely have occurred even more rapidly, and plasma virus titers reached higher levels, had not CTL responses directed against other viral gene products developed.

HIV-1 variants which are not recognized by autologous CTL have previously been observed in longitudinal studies of HIV seropositive patients (74-76), but it has generally been difficult to demonstrate that CTL escape mutant viruses have a clear selective advantage *in vivo*. One factor that may have facilitated the demonstration of CTL escape variants in this study may be our approach for identifying changes in the viral quasispecies by analyzing plasma viral RNA (cDNA). Plasma virus exhibits a circulating half-life ($T_{½}$) of approximately six hours (73) and the cells producing most of this virus a $T_{½}$ of approximately 2 days (54, 72). However, these virus-producing cells are under-represented in the blood where latently infected cells

and defectively-infected cells (with half-lives as long as 80 days) predominate (54). Analysis of plasma viral RNA gives a dynamic assessment of the most active viral compartments.

Immunologically, the fact that the earliest CTL response in patient WEAU was predominantly focused on a highly immunodominant viral epitope and that the CTLp frequency was so high early after infection also may explain why CTL-mediated selection of escape-conferring mutations was more evident in this patient than in previous studies (74-78). If CTL pressure is simultaneously directed against several codominant epitopes, the outgrowth of virus variants with escape-conferring mutations in only one of these epitopes may be controlled by CTL directed against the other epitopes, unless the variant epitopes have strongly antagonistic properties (79-81). At 16 DFOSx, limiting dilution assays showed the frequency of epitope-specific CTLp to be approximately 1 per 17 PBMC and even this value is likely to be an underestimate since the PBMC had been cryopreserved prior to testing. This frequency is similar to the CTLp frequencies measured at the peak of the acute response to LCMV infection in mice (69,82). The primary CTL response in patient WEAU may thus have been near its peak around 16 DFOSx. Whether HIV-1 specific CTLp generally reach such high frequencies at the peak of the early immune response is currently unclear.

As patient WEAU obviously had the capacity to mount CTL responses to multiple HIV-1 epitopes, it remains unclear why the earliest CTL response in this patient was so predominantly focused against the gp160 aa 30-38(9) epitope. One possibility is that prior unrelated infections in this patient left him with a population of memory T cells which crossreacted on this epitope and that these cells, being present at higher frequency and more readily activated than naive T cells directed against other HIV-1 epitopes, dominated the initial HIV-1-specific immune response. That the CTL response mounted to a virus infection may be modified by the host's prior immune experience with unrelated pathogens has been demonstrated in murine virus infections (83). Alternatively (or in addition), the gp160 aa 30-38(9) epitope may have been particularly immunogenic. In this context, it is of interest that during the natural processing of gp160, signal

peptide cleavage occurs between aa 29 and 30, thus generating the same N-terminus as the aa 30-38 CTL epitope. This may have favored production of the aa 30-38 peptide at higher levels than other epitopic peptides, and resulted in preferential presentation of this epitope especially at very early times after infection when levels of viral antigens were limiting. The immunogenicity of a peptide is also influenced by its affinity of binding to MHC and the affinity of peptide-MHC complexes for the T cell receptor. The gp160 aa 30-38(9) epitope may have been "strong" in these respects, and the epitopes recognized later may have been "weaker". The shift in the response to "weaker" epitopes may have reduced the long-term efficiency of containment of virus replication (76) and been one of the factors which contributed to the subsequent rapid rate of disease progression in patient WEAU (84). Recent studies (85), suggest that the viral load established early in HIV infection is a predictor of the subsequent clinical course, with higher viral loads after seroconversion predicting shorter survival. The shift in CTL specificity which occurred around the time of seroconversion in patient WEAU may also have influenced the viral "setpoint" established in this patient, again contributing to the rapid rate of disease progression he underwent.

Future in-depth analysis of virus-CTL interactions in larger numbers of patients is needed to reveal how commonly CTL escape virus variants are selected for during acute HIV-1 infection, and whether their selection is correlated with the establishment of a high viral "setpoint" and a rapid rate of subsequent disease progression. Preliminary results we have obtained in a second patient show that here too, rapid selection occurred within the plasma virus population for mutants bearing aa changes in a gp160 epitope recognized by the early CTL response. Interestingly, this patient was also a rapid disease progressor. The events we report here in patient WEAU are thus not unique and may in fact prove to represent a mechanism that HIV-1 commonly uses to evade control by the early antiviral immune response.

CONCLUSIONS

The studies reported here provide the first conclusive demonstration of plasma virus load as an indicator of HIV-1 replication dynamics in infected tissues and the first estimate of lifespans of plasma virus

and virally infected cells. Furthermore, the results presented provide the first direct demonstration of the substantial and biologically relevant pressure exerted by the early $CD8^+$ CTL response on HIV-1 replication *in vivo*. Together with suggestive evidence already in the literature that virus-specific $CD8^+$ CTL make an important contribution to containment of virus during chronic infection, these findings suggest that stimulation of this arm of the immune response should be an important goal of future prophylactic and therapeutic strategies to combat HIV-l infection.

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Table 1: Comparison of Virologic Assay Detection Rates in Different Stages of HIV-1 Infection*

Site	CD4 Count	Number Tested	Plasma Culture	p24 Ag		Plasma Viral RNA	
				Regular	ICD	QC-PCR	bDNA
ADARC	<200	52	45(87)▲	26(50)	ND	ND	47(90)
	200-500	18	6(33)	3(17)	ND	ND	14(78)
	>500	8	0(0)	0(0)	ND	ND	6(75)
	Subtotal	78	51(65)	29(37)	ND	ND	67(86)
UAB	<200	31	24(77)	26(84)	27(87)	31(100)	30(97)
	200-500	19	5(26)	6(32)	8(42)	19(100)	15(79)
	>500	16	0(0)	1(6)	3(19)	16(100)	11(69)
	Subtotal	66	29(44)	33(50)	38(58)	66(100)	56(85)
All Sites	Total	144	80(56)	62(43)	38(58)	66(100)	123(85)

* Patients with primary (acute) HIV-1 infection were excluded from this analysis.

▲ Number and (percentage) of subjects with positive assay results.

Table 2: Spearman Rank Correlations Among Viral Load Measurements and CD4+ Lymphocyte Counts*

	CD4+ Cells	p24 Ag	ICD p24 Ag	Plasma Culture	QC-PCR	bDNA
CD4+ Cells	–	-.58	-.61	-.67	-.75	-.72
p24 Ag		–	.90	.62	.52	.51
ICD-p24 Ag			–	.58	.54	.56
Plasma Culture				–	.76	.72
QC-PCR					–	.89
bDNA						–

*Comparisons performed on viral load data depicted in Figure 1 along with corresponding p24 Ag data and CD4+ lymphocyte counts. Patients with primary (acute) infection and those with negative bDNA values were excluded.

All correlations shown are significant at the $p < 0.0001$ level.

Subject	Specimen		Functional Clones	NVP-Sensitive Clones	NVP-Resistant Clones
1625	Plasma	day-7	80	80 (100%)	0 (0%)
		+14	72	27 (38%)	45 (62%)
		+28	57	0 (0%)	57 (100%)
		+84	67	0 (0%)	67 (100%)
		+140	86	0 (0%)	86 (100%)
1625	PBMC	-7	163	163 (100%)	0 (0%)
		+14	121	121 (100%)	0 (0%)
		+28	258	134 (52%)	124 (48%)
		+84	133	43 (32%)	90 (68%)
		+140	261	65 (25%)	196 (75%)
1624	Plasma	-7	19	19 (100%)	0 (0%)
		+14	34	4 (12%)	30 (88%)
		+28	79	6 (8%)	73 (92%)
		+140	27	0 (0%)	27 (100%)
1624	PBMC	-7	24	24 (100%)	0 (0%)
		+14	34	29 (85%)	5 (15%)
		+28	52	42 (81%)	10 (19%)
		+140	87	26 (30%)	61 (70%)
1605	PBMC	-7	31	31 (100%)	0 (0%)
		+140	31	11 (35%)	20 (65%)
1619	Plasma	-14	79	79 (100%)	0 (0%)
		+28	41	0 (0%)	41 (100%)
		+140	38	0 (0%)	38 (100%)

TABLE 3. *In situ* functional analysis of HIV-1 reverse transcriptase genes. Full-length RT genes were amplified by PCR from uncultured plasma and uncultured PBMCs. DNA products were cloned into the *Eco*RI and *Hin*dIII sites of the bacterial expression plasmid pLG18-1. The expression plasmids were screened for the presence of functional RT and tested *in situ* for susceptibility to NVP inhibition at 3000 nM concentration (approximately 50-75 fold greater than the IC_{50}). To ensure accuracy in distinguishing RT genes which encoded NVP-resistant versus sensitive enzymes, and to confirm the identification of specific NVP resistance-conferring RT mutations obtained by the direct sequencing method, we determined the complete nucleotide sequences of 21 cloned RT genes which had been phenotyped in the *in situ* assay. There was complete concordance between the phenotypes and genotypes of these 21 clones with respect to NVP resistance-conferring mutations as well as complete concordance between direct viral population sequences and clone-derived sequences at NVP resistance-conferring codons.

Table 4 Clinical Course of Patient WEAU

DFOSx*	CD4	HIV-1 Ab ■		Plasma Viremia†		
		ELISA	WB	Infectivity	RNA	p24 Ag
15	358	-	-	1000	216,400	80
16		-	-	1000		103
20		+	-	1	355,200	299
23		+	+	0	355,400	258
27	748	+	+	0	146,800	32
34		+	+	0	100,900	11
44	972	+	+	0	34,700	0
72		+	+	0	11,400	0
136		+	+	0	17,322	0
212	197	+	+	0	90,109	0
391	89	+	+	5	55,268	0

*DFOSx - days following onset of symptoms of the acute retroviral syndrome.

■ Presence (+) or absence (-) of HIV-1 specific antibody as determined by enzyme-linked immunosorbent assay (ELISA) or Western Blot (WB).

†Plasma viremia as determined by infectivity titers (tissue culture infectious doses per milliliter of plasma; ref 19); plasma viral RNA (molecules per milliliter; ref 23); and HIV-1 p24 core antigen after antigen-antibody dissociation (pg per milliliter; ref 23).

'CD4 counts declined to 30/mm^3 772 DFOSx and to 51 mm^3 1099 DFOSx. The patient received antiretroviral therapy beginning 540 DFOSx but died 1601 DFOSx.

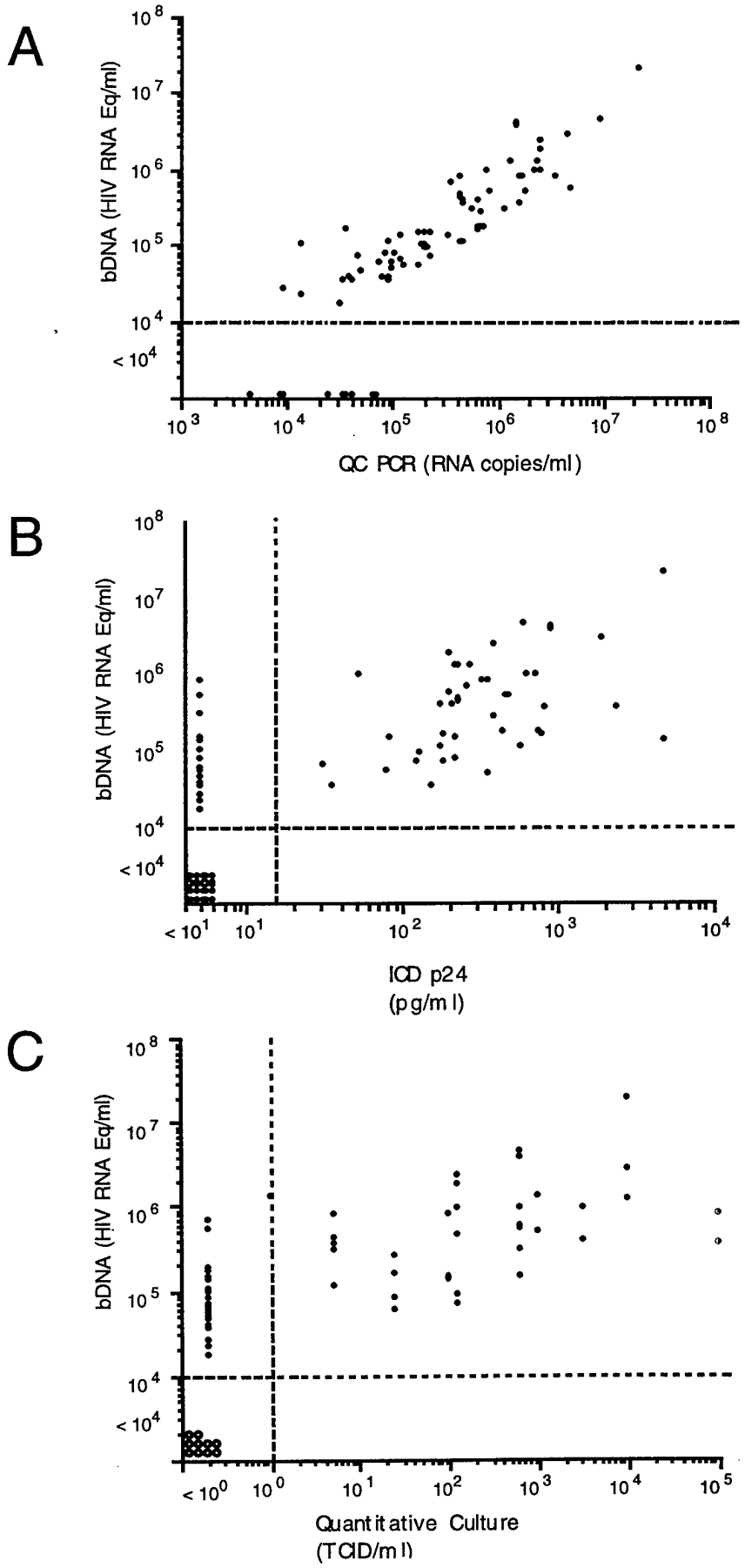


Figure 1: Plasma viral RNA levels determined by bDNA assay compared with plasma viral RNA assayed by QC-PCR (Panel A), plasma viral p24 Ag determined by the immune complex dissociation (ICD) assay (panel B), and infectious plasma virus determined by endpoint culture (panel C). Sensitivity cutoff levels for the respective assays are represented by dashed lines.

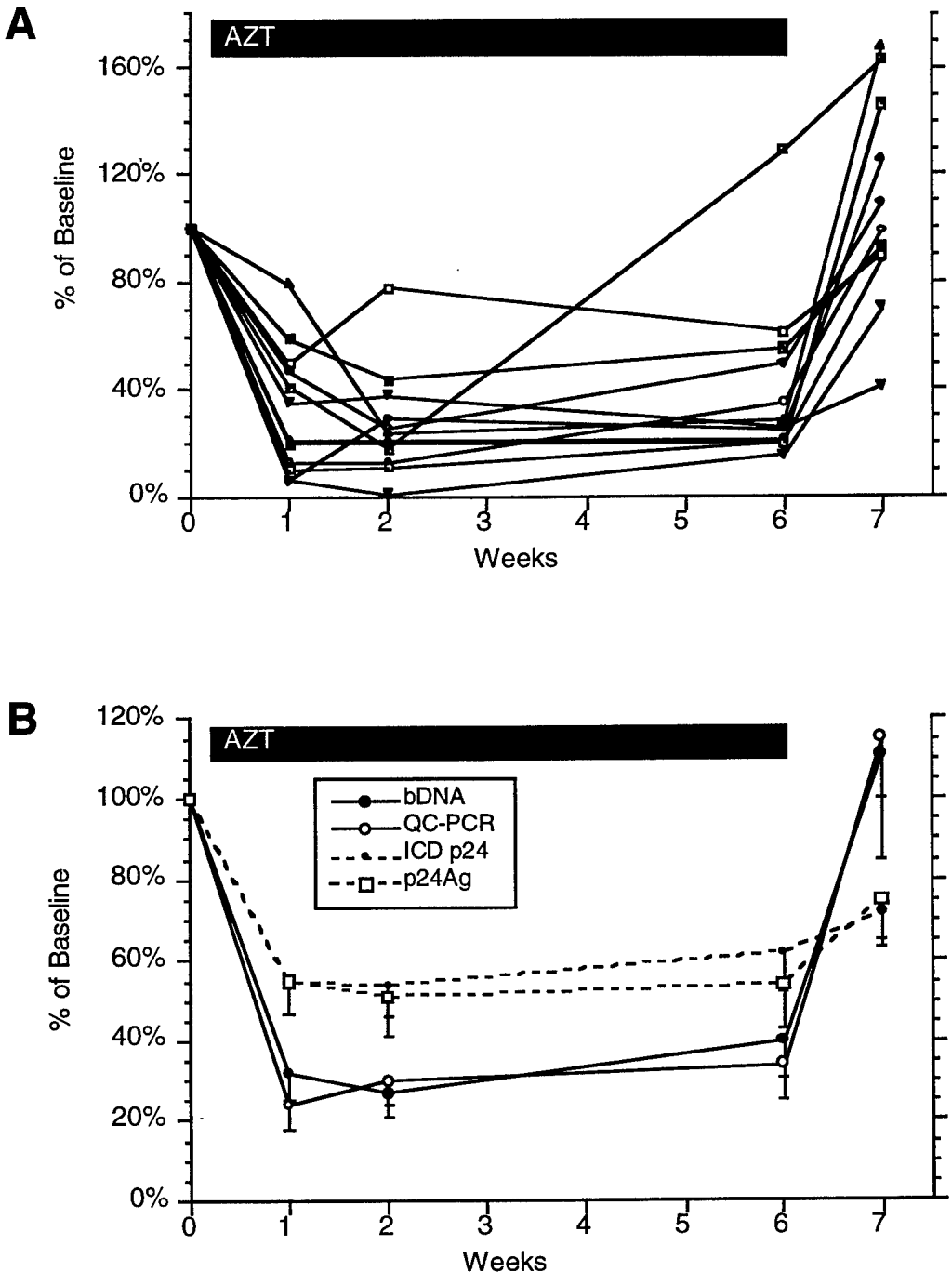


Figure 2: Plasma viral load measurements by bDNA, p24 Ag, and endpoint culture assays in two patients (panels A and B) with primary (acute) HIV-1 infection. The timing of first antibody detection and seroconversion are indicated by the bar labelled seroconversion. Note that bDNA values as plotted have been multiplied by 10^{-3}, so that a value plotted at 10^3 actually represents 10^6 HIV-1 RNA Eq/ml.

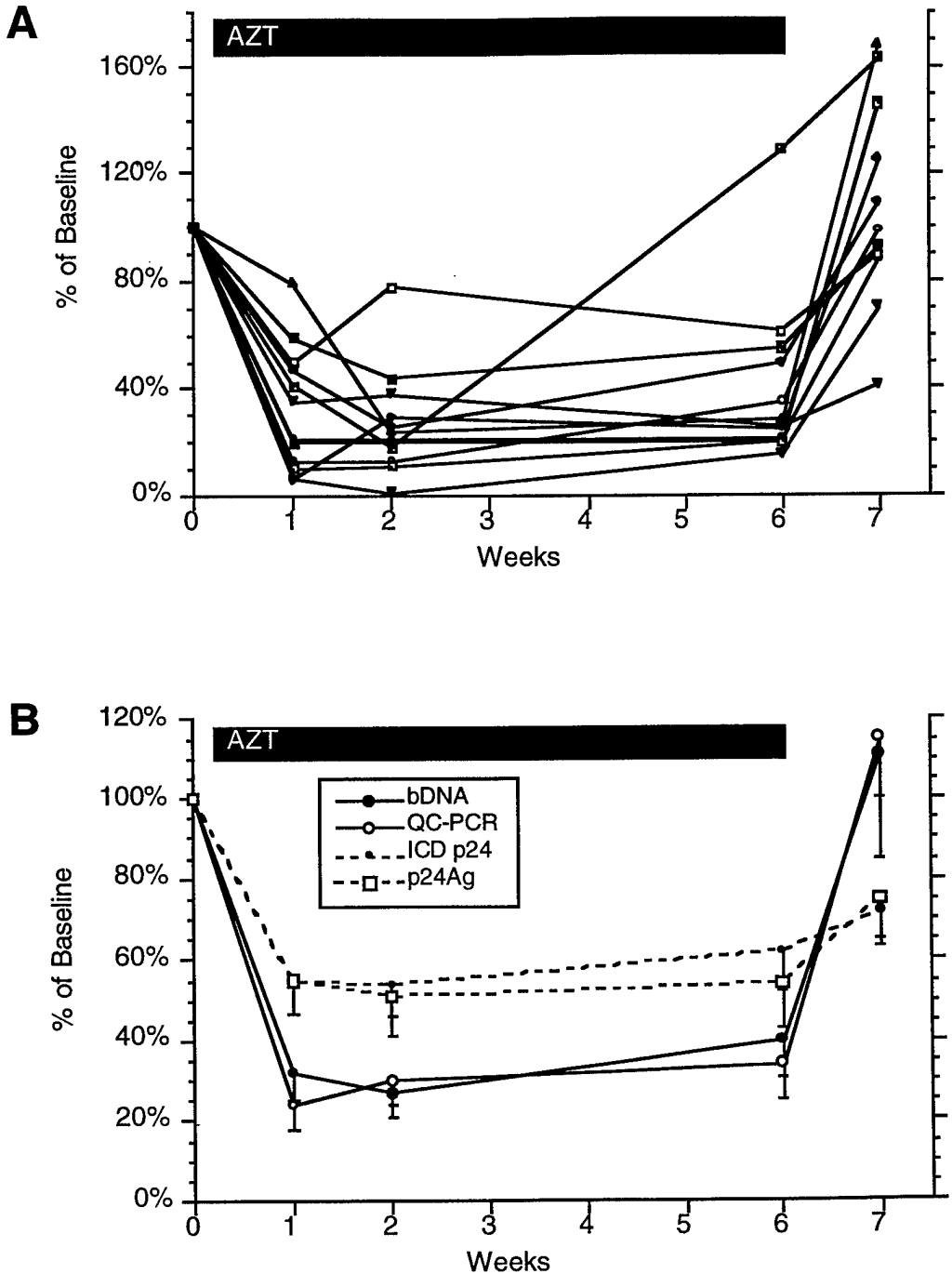


Figure 3: Changes in plasma viral RNA determined by bDNA and QC-PCR assays and plasma viral core antigen determined by regular and ICD-p24 Ag assays following the institution of a six week course of zidovudine (AZT) at 500 mg qd. Therapy was discontinued after week six. Viral load measurements are plotted as percent of baseline (mean ± 1 S.E.M.). Individual patient data for the bDNA assay are shown in panel A (n=12) and combined data (mean ± 1 S.E.M.) are shown in panel B. Baseline values for viral RNA determined by bDNA assay ranged from 4.8 X 10^4 to 7.9 x 10^5 Eq/ml, and by QC-PCR assay, from 3.5 X 10^4 to 1.2 x 10^6 RNA molecules/ml. Baseline p24 Ag ranged from 0-226 pg/ml and ICD-p24 Ag from 0-783 pg/ml.

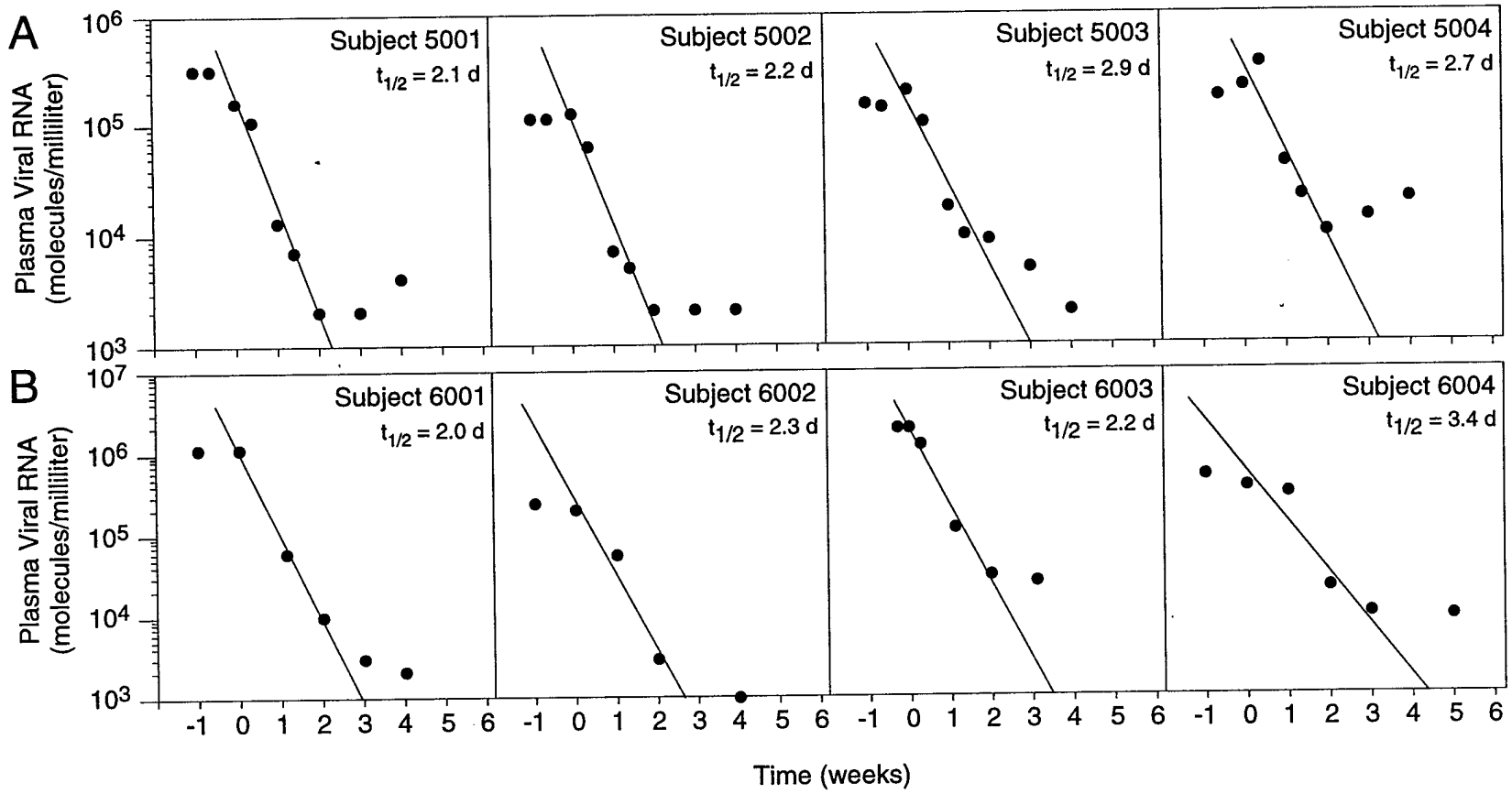


FIG 4. Plasma viral RNA determinations in representative subjects treated with the HIV-1 protease inhibitors ABT-538 (panel A) and L-735, 524 (panel B). METHODS. Subjects had not received other antiretroviral agents for at least 4 weeks prior to therapy. Treatment was initiated at week 0 with 400-1200 mg/d of ABT-538 or 1600-2400 mg/d of L-735,524 and was continued throughout the study. Viral RNA determinations were performed by modified bDNA (panel A) or RT-PCR (panel B) assays and confirmed by QC-PCR. Shown are the least squares fit linear regression curves for data points between days 0 and 14 indicating exponential (first-order) viral elimination.

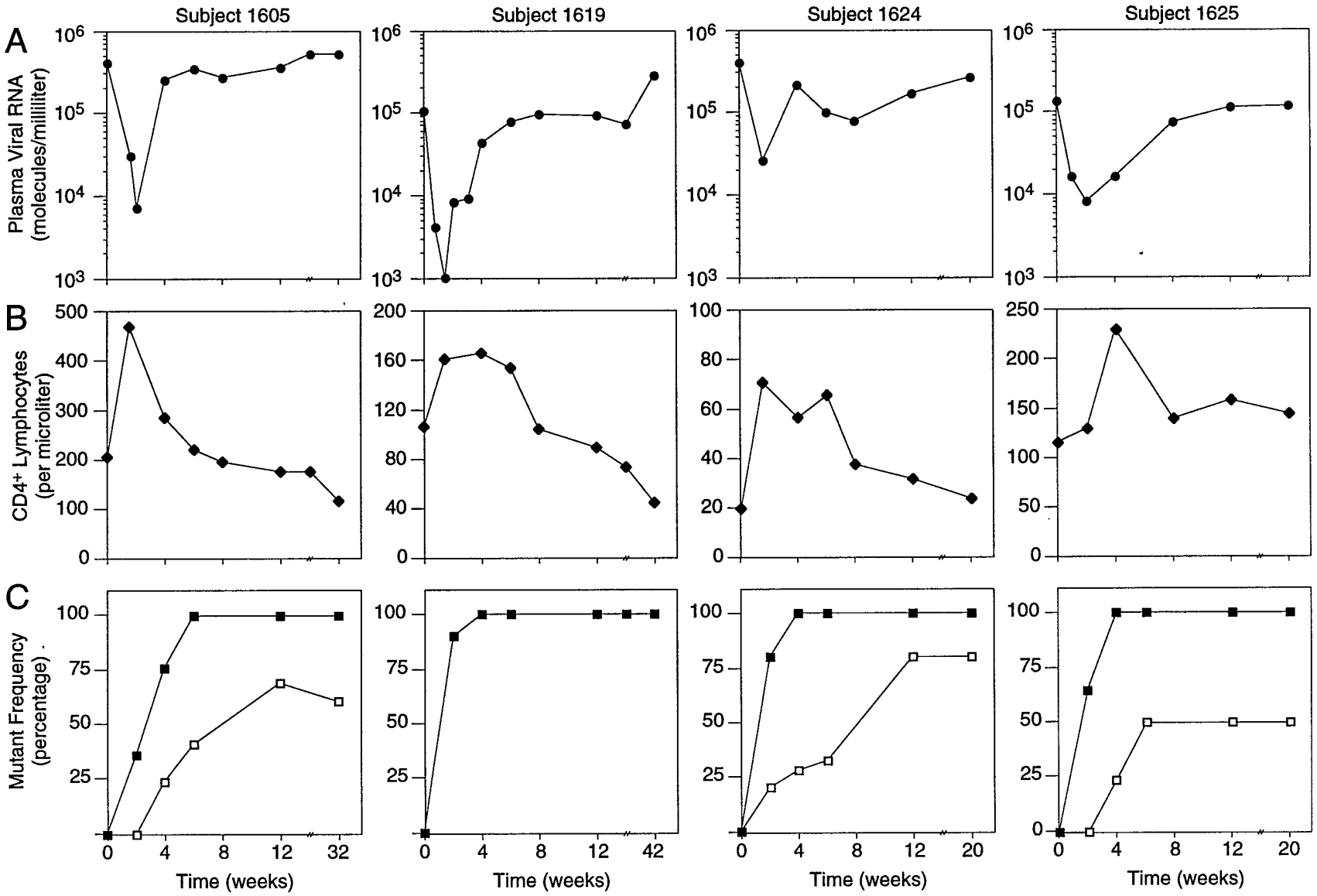


Fig. 5. Plasma viral RNA determinations (panel A), CD4+ lymphocyte counts (panel B), and percentages of mutant viral genomes in plasma and PBMCs (panel C) of subjects initiating treatment with NVP. Subjects were participants in a clinical protocol assessing the effects of NVP when added to existing treatment with ddI (subject 1605) or ddI + zidovudine (subjects 1619, 1624, 1625). Treatment with NVP was initiated at week 0 using 200 mg/day and was increased to 400 mg/day after 2 weeks. ddI and zidovudine dosages were 400 mg/day and 300-600 mg/day, respectively. Viral RNA determinations (-●-) were performed by QC-PCR assay. CD4+ lymphocytes (-♦-) were quantified by flow cytometry. Frequencies of viral genomes containing NVP resistance-associated mutations in plasma (-■-) and PBMCs (-□-) were determined by automated DNA sequence analysis, with each data point representing the average of 3-6 independent determinations.

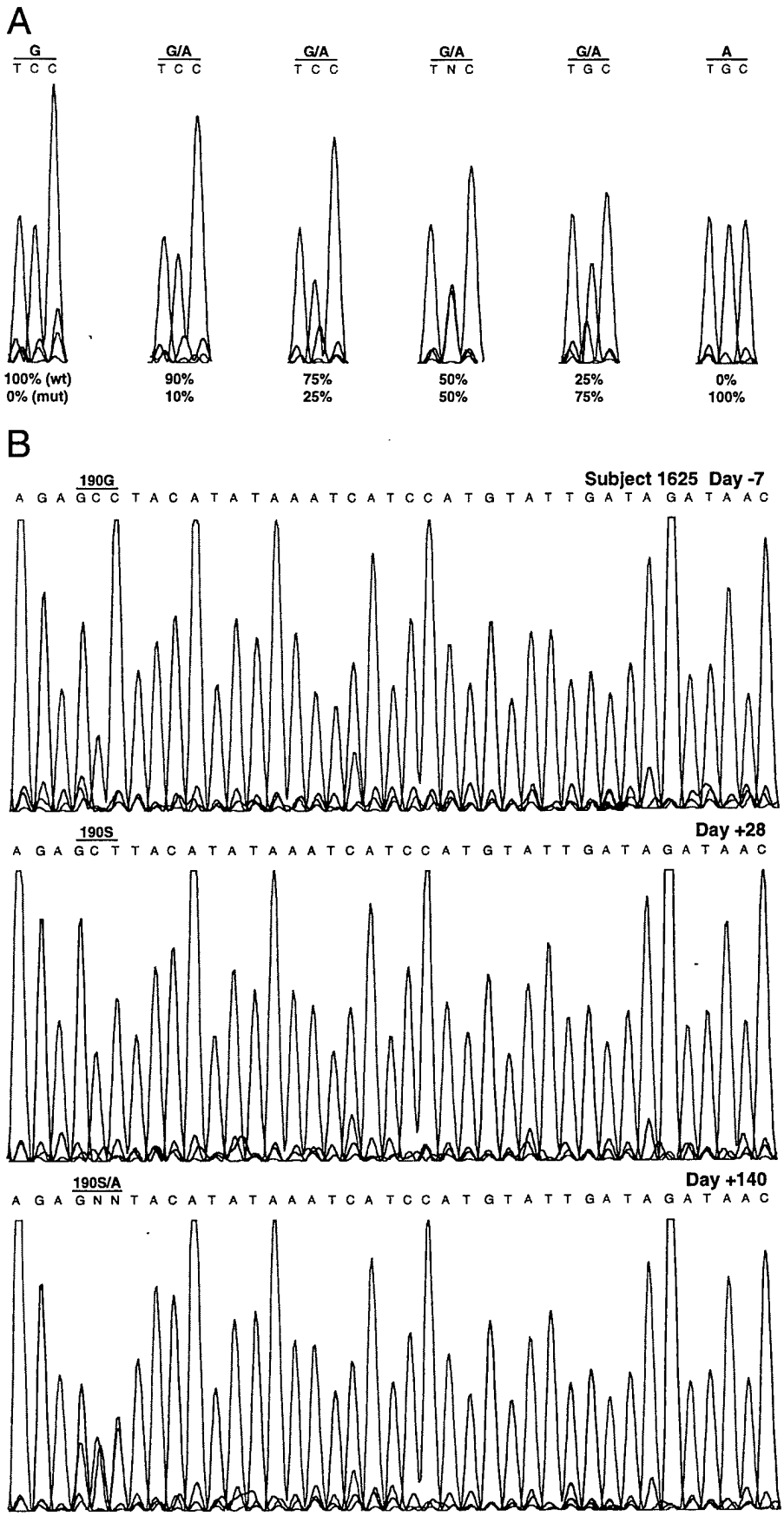


FIG 6 (Legend follows on next page)

FIG 6. Quantitative detection of HIV-1 drug resistance mutations by automated DNA sequencing. Panel A: DNA sequence chromatograms of RT codon 190 from a defined mixture of wild-type (wt) and mutant (mut) HIV-1 cDNA clones differing only at the second base position of the codon. Sequences shown were obtained from, and therefore are presented as, the minus (non-coding) DNA strand. For example, the minus strand TCC sequence shown corresponds to the plus strand codon GGA (glycine, G). Similarly, the minus strand TGC sequence corresponds to the plus strand codon GCA (alanine, A). The single letter amino acid code corresponds to the plus strand DNA sequence. Mixed bases approximating a 50/50 ratio are denoted N. Panel B: DNA sequence chromatograms of RT codons 179-191 (again displayed as the minus strand sequence) derived from plasma virion-associated RNA of subject 1625 before (day -7) and after (days +28 and +140) the initiation of NVP therapy. Codon changes resulting in amino acid substitutions at position 190 are indicated for the plus strand. For example, the GCC minus strand sequence at position 190 (day -7) corresponds to GGC (glycine, G), and the GCT minus strand sequence at position 190 (day +28) corresponds to AGC (serine, S) in the respective plus strands. Mixtures of wt and mut cDNA clones (panel A) were prepared and diluted such that first round PCR amplifications were performed with 1000 viral cDNA target molecules per reaction. HIV-1 RNA was isolated from virions pelleted from *uncultured* plasma specimens (panel B). cDNA was prepared using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) and an oligonucleotide primer corresponding to nucleotides 4283 to 4302 of the HXB2 sequence. The full-length viral reverse transcriptase gene (1680 bp) was amplified by means of a nested polymerase chain reaction (PCR) using conditions and oligonucleotide primers (outer primers: nt 2483-2502 and 4283-4302; inner primers: nt 2549-2565 and 4211-4229). Subgenomic fragments of the RT gene were also amplified using combinations of the following oligonucleotide primers: (5') 2585-2610; (5') 2712-2733; (3') 2822-2844; (3') 3005-3028; (3') 3206-3228; (3') 3299-3324; (3') 3331-3350; (3') 3552-3572; and (3') 3904-3921. All 3' primers incorporated the universal primer sequence for subsequent dye-primer sequence analysis. The HIV-1 copy number in every PCR reaction was determined (100-10,000 copies). A total of three to six separate PCR amplifications of primary patient material was performed on each sample using different combinations of primers and representative chromatograms are shown. Rarely, codon interpretation was ambiguous. In the day +140 plasma sample from subject 1625 (bottom of panel 3B), the complementary (plus) strand could read: AGC(serine), GCN(alanine), ACN(threonine), AGA/AGG(arginine), or GGN(glycine). In this case, we sequenced 7 full-length RT molecular clones and found them to encode only serine or alanine. Sequencing was performed using an automated ABI 373A sequenator and the Taq Dye Primer Cycle Sequencing Kit (ABI). Sequence analysis was performed using Sequencher (Gene Codes Corp.) and Microgenie (Beckman) software packages, and base pair mixtures were quantified by measuring relative peak-on-peak heights.

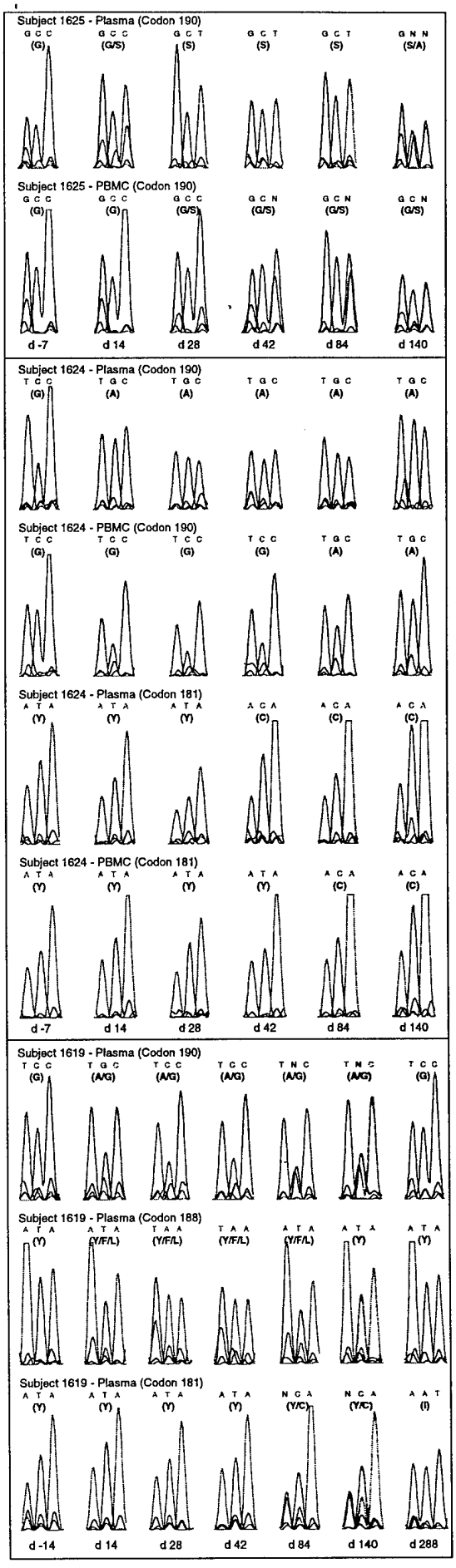


FIG 7. Quantitative detection of HIV-1 drug resistance mutations by automated DNA sequencing in plasma viral RNA (cDNA) and PBMC-associated viral DNA populations before and after the initiation of NVP on day 0. Minus strand sequences are shown along with single letter amino acid codes of the corresponding plus strand sequence. Mixed bases approximating a 50/50 ratio are denoted N. HIV-1 cDNA was prepared from virions pelleted from uncultured plasma as described. Viral DNA was isolated from uncultured PBMCs. The full-length viral reverse transcriptase genes as well as subgenomic fragments were amplified and sequenced. The HIV-1 copy number in every PCR reaction was determined (100-10,000 copies). A total of three to six separate PCR amplifications of primary patient material was performed on each sample using different combinations of primers and representative chromatograms are shown. Some sequences were determined from both coding and non-coding DNA strands to ensure the accuracy of quantitative measurements.

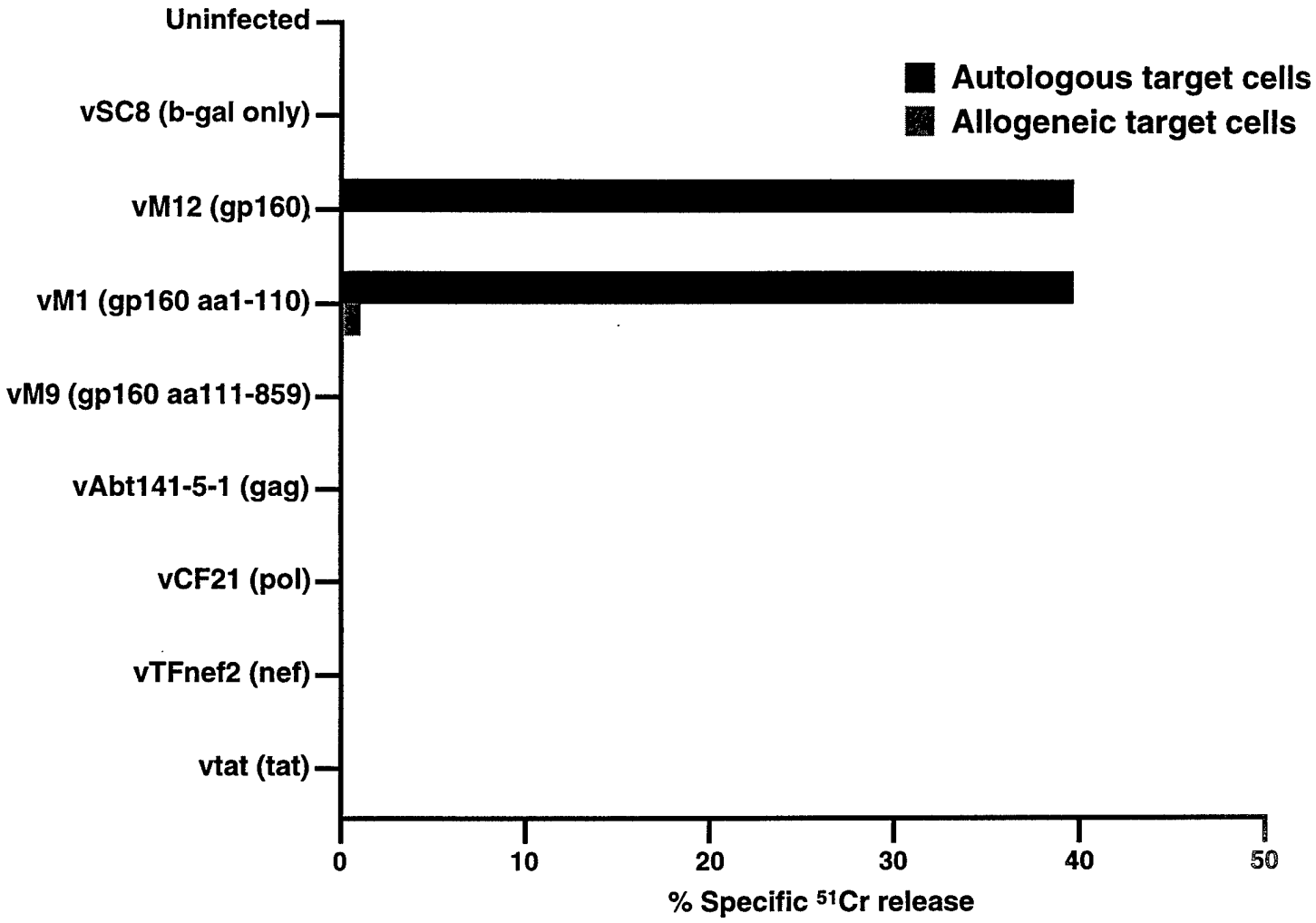
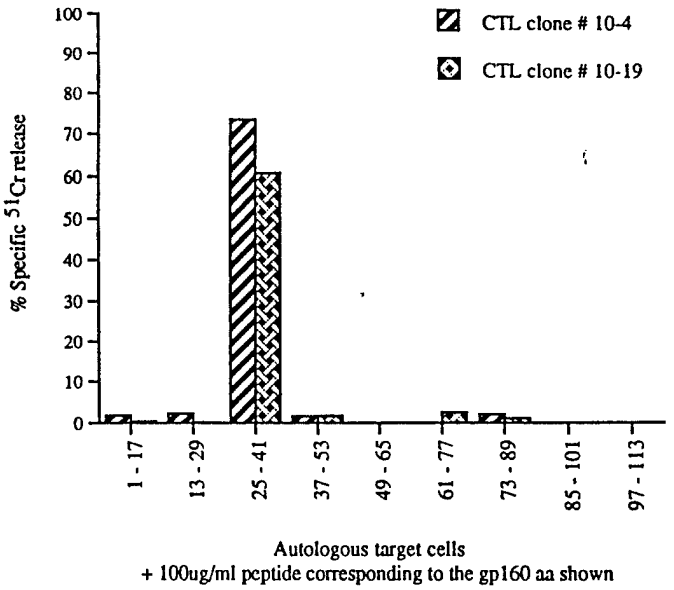
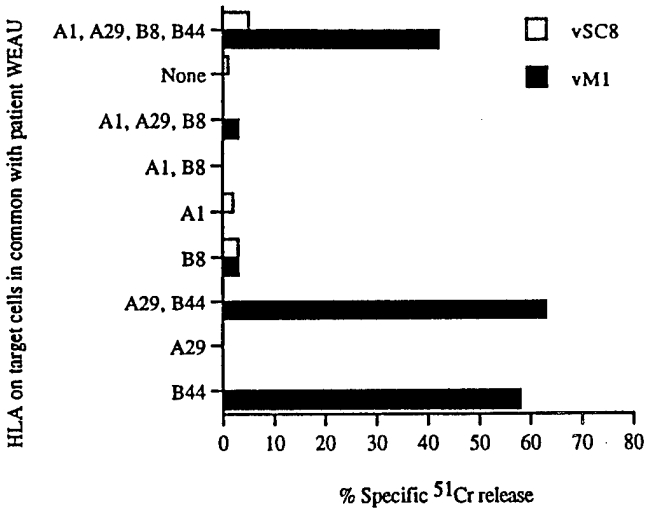


Figure 8. HIV-1-specific CTL activity mediated by PBMC cryopreserved from patient WEAU 20 DFOSx directed against epitope(s) in the first 110 aa of gp160. The results shown are the % specific ^{51}Cr release mediated by *in vitro* restimulated polyclonal CTL at an effector: target cell (E:T) ratio of 40:1 from autologous and allogeneic EBV-B-LCL target cells infected with recombinant vaccinia viruses encoding β-galactosidase (b-gal) only (vSC8); full-length gp160 (vM12) or sections thereof (vM1 and vM9) derived from the autologous virus in this patient 15 DFOSx; or other HIV-1 proteins (vAbT 141-5-1, vCF21, vTFnef2 and vtat) as indicated.

a



b



c

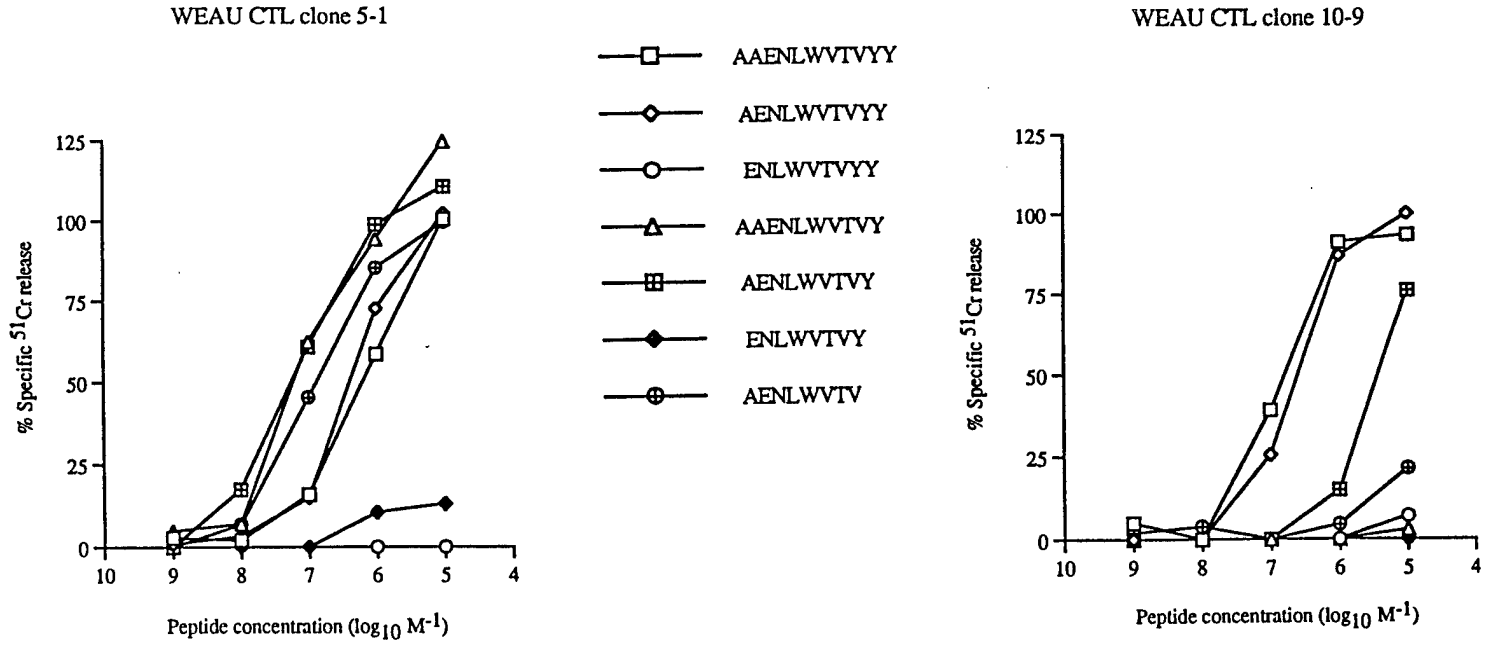
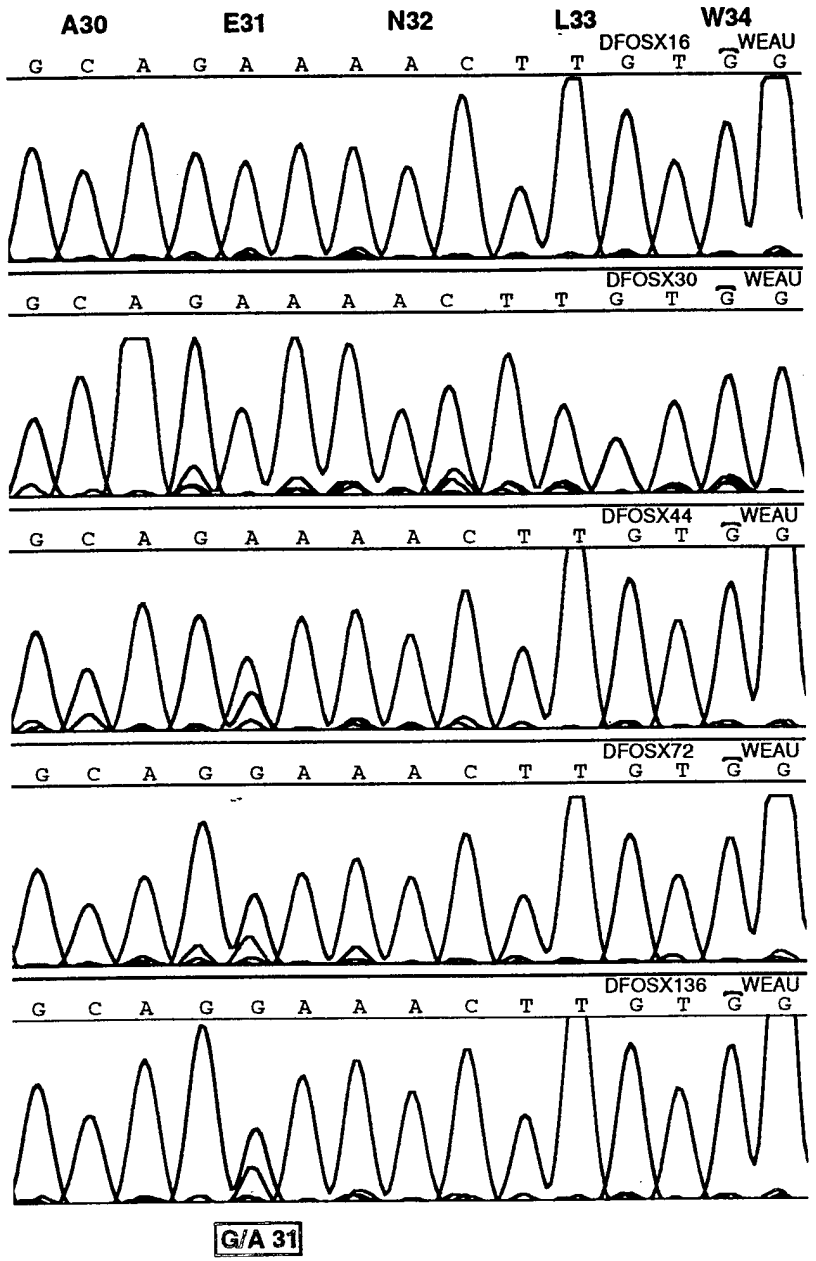


Figure 9. Epitope mapping studies showing that CTL clones derived from patient WEAU 20 DFOSx recognize gp160 aa 30-38(9) in association with HLA-B44. *a*, % specific ^{51}Cr release mediated by two CTL clones at an E:T ratio of 10:1 from autologous EBV-B-LCL target cells in the presence of 100 μg/ml of a series of synthetic peptides (each 17 aa long and overlapping by 5aa) corresponding to the gp160 sequence of the autologous virus of patient WEAU 15 DFOSx. The aa 25-41 peptide recognized by both clones (and others tested) has the sequence MICSAAENLWVTVYYGV. *b*, lysis mediated by CTL clone #10-4 at an E:T ratio of 10:1 of autologous EBV-B-LCL target cells (HLA-A1, A29; B8, B44) and EBV-BLCL sharing between 0 and 3 HLA-A or B molecules with patient WEAU as indicated, following infection with recombinant vaccinia viruses vM1 (expresses autologous gp160 aa 1-110) or vSC8 (expresses b-gal only). Target cells sharing HLA-B44 with patient WEAU were recognized by this and other day 20 WEAU CTL clones (not shown) after infection with vM1. *c*, % specific ^{51}Cr release mediated by two CTL clones at an E:T ratio of 10:1 from autologous target cells in the presence of different concentrations of the synthetic peptides shown, which represent the gp160 aa 29-39 sequence of the autologous virus of patient WEAU 15 DFOSx (AAENLWVTVYY), and amino or carboxy-terminal truncated versions of this peptide. The optimal epitope length for recognition by the two clones differs, clone #5-1 preferring (A)AENLWVTVY, whilst clone #10-9 prefers (A)AENLWVTVYY.



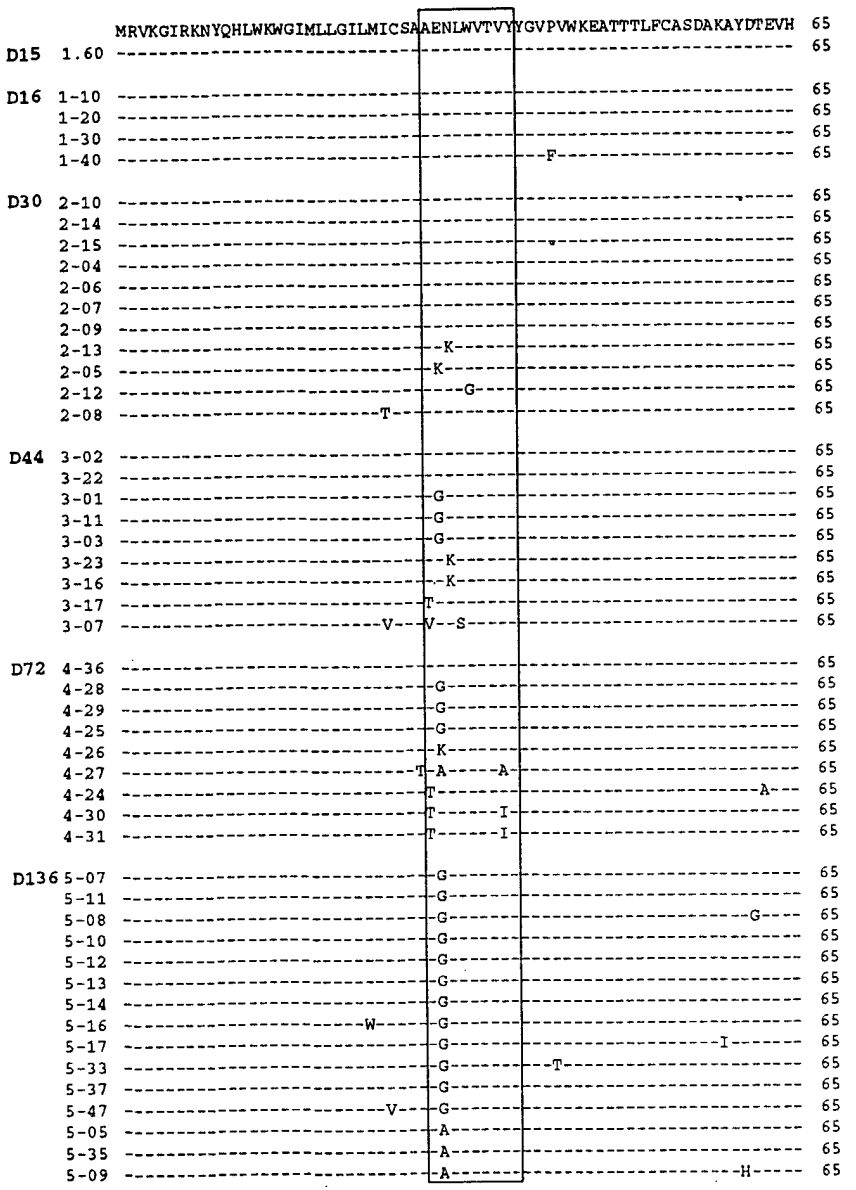


Figure 10. Quantitative detection of HIV-I CTL escape mutations by automated sequence analysis of plasma viral RNA (cDNA). *a*, population sequences of codons 30-34 of the gp160 gene are shown, demonstrating the emergence of mutant virus [GAA(**E**) to GAA/GGA (**E/G**) at codon 31] on day 44 and with complete replacement of wild-type virus (AENLW) by mutant virus (AGNLW/AANLW) by day 136. *b*, deduced amino acid sequences of individual plasma viral RNA (cDNA) clones corresponding to the amino-terminal 65 aa of gp160. The CTL epitope at position 30-38 is boxed.

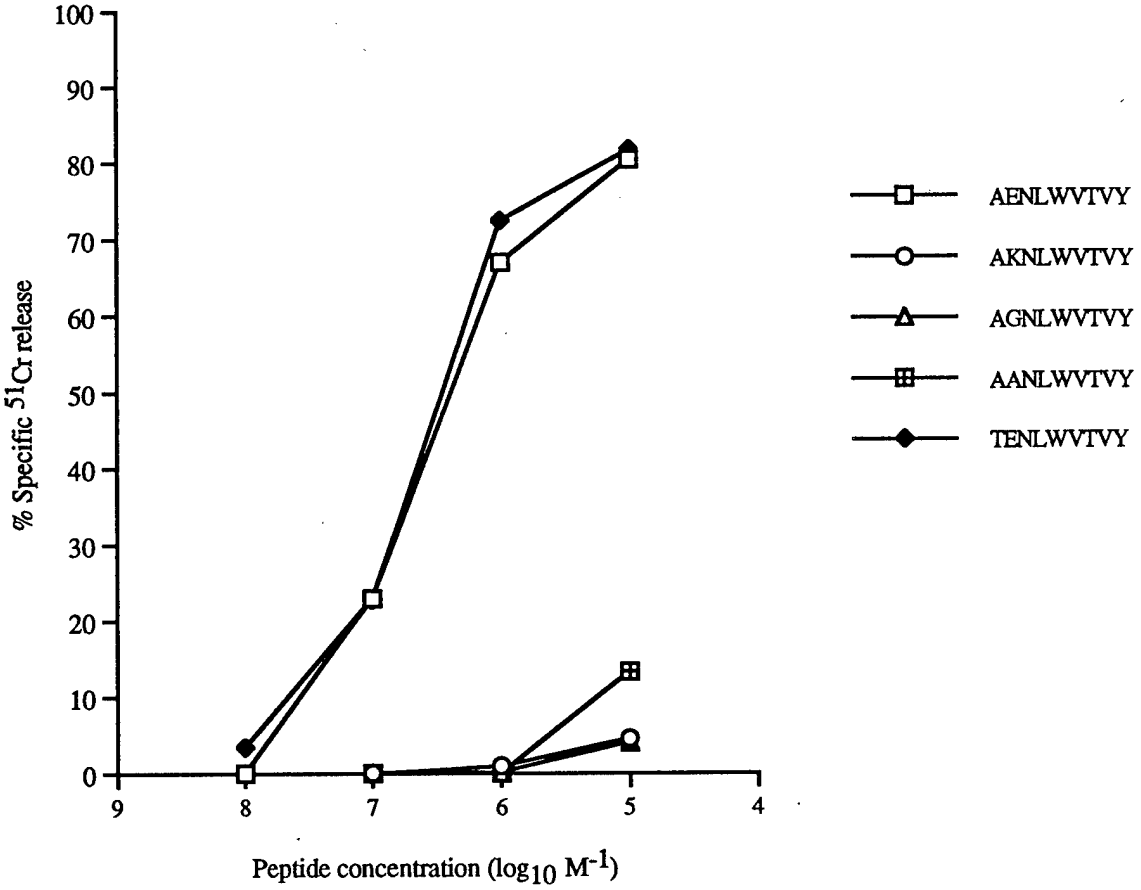


Figure 11. Relative abilities of synthetic peptides corresponding to gp160 aa 30-38 of the virus population in patient WEAU 16 DFOSx, and of the predominant mutant populations which emerged later, to sensitize autologous target cells for lysis by early WEAU CTL. The results shown are the % specific ^{51}Cr release mediated by day 20 WEAU CTL clone #5-1 at an E:T ratio of 10:1 from autologous EBV-B-LCL target cells in the presence of different concentrations of synthetic peptides, as indicated.

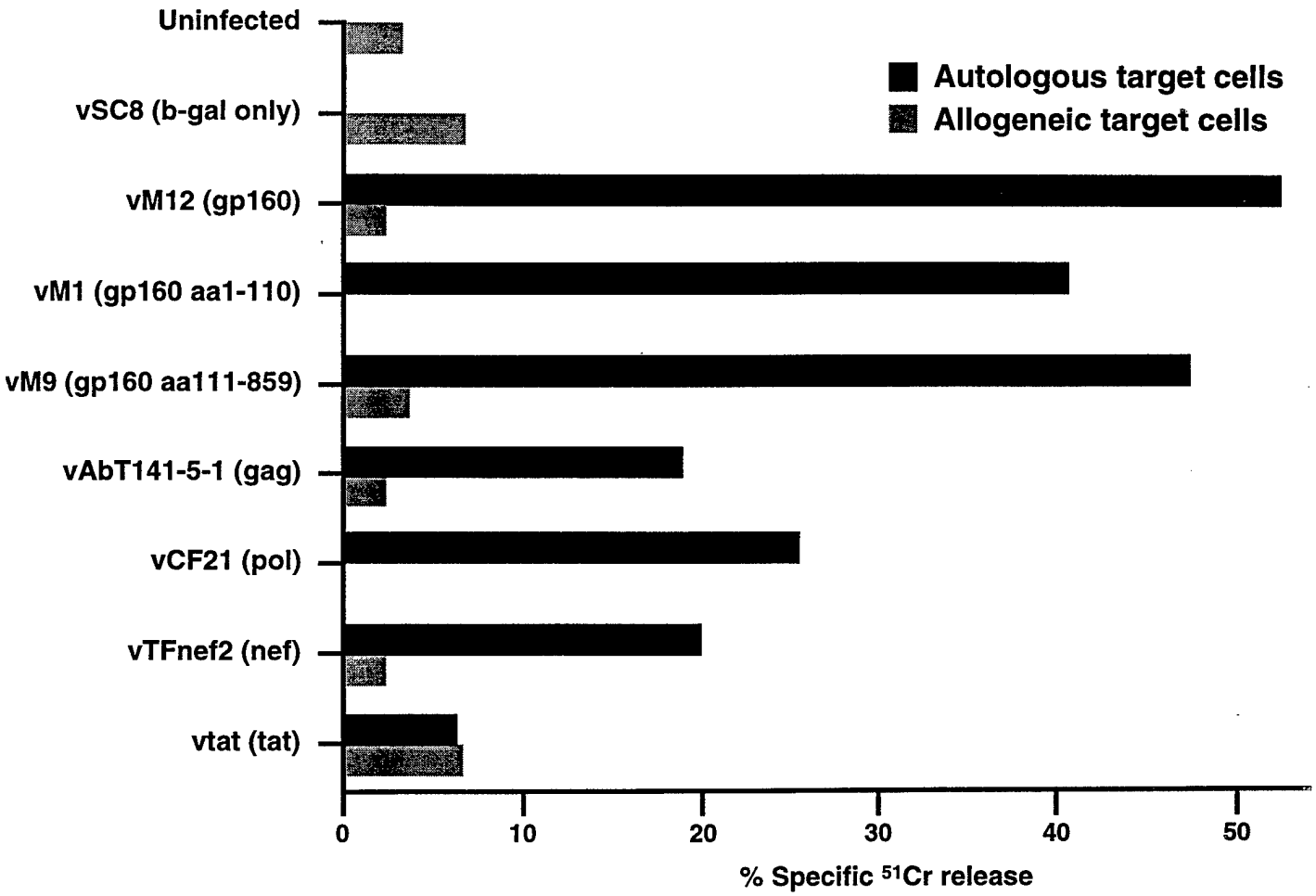


Figure 12. PBMC cryopreserved from patient WEAU 30 DFOSx mediate CTL activity directed against multiple HIV-1 epitopes. The results shown are the % specific ^{51}Cr release mediated by *in vitro* restimulated polyclonal CTL at an E:T ratio of 40:1 from autologous and allogeneic EBV-B-LCL target cells infected with recombinant vaccinia viruses encoding b-gal only (vSC8); gp160 derived from the plasma virus in patient WEAU 15 DFOSx and sections thereof (vM12, vMl and vM9); or other HIV-1 proteins (vAbT 141-5-1, vCF21, vTFnef2 and vtat) as indicated.

Appendix

PUBLICATIONS

Saag, M.S., E.A. Emini, O.L. Laskin, J. Douglas, W.I. Lapidus, W.A. Schleif, R.J. Whitley C. Hilderbrand, V.W. Byrnes, J.C. Kappes, K.W. Anderson, F.E. Massari, and **G.M. Shaw**: A short-term clinical evaluation of L-697,661, a non-nucleoside inhibitor of HIV-1 reverse transcriptase. *New England Journal of Medicine* 329:1065-1072, 1993.

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PERSONNEL RECIEVING PAY FROM THIS CONTRACT

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Radding, Wilson
Taylor, Maria
Wei, Xiping
Decker, Julie
Hill, Sherri Campbell
Niu, Yafen
Zhang, Yi
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A SHORT-TERM CLINICAL EVALUATION OF L-697,661, A NON-NUCLEOSIDE INHIBITOR OF HIV-1 REVERSE TRANSCRIPTASE

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Abstract *Background.* The non-nucleoside reverse transcriptase inhibitors are novel antiretroviral agents with selective activity in vitro against human immunodeficiency virus type 1 (HIV-1). They act through direct inhibition of reverse transcriptase and are not incorporated into DNA.

Methods. We evaluated a pyridinone non-nucleoside reverse transcriptase inhibitor, L-697,661, in separate six-week double-blind trials in patients with HIV-1 infection whose CD4 counts ranged from 200 to 500 cells per cubic millimeter (68 patients) or less than 200 cells per cubic millimeter (67 patients). Eligible patients were randomly assigned to receive L-697,661 orally in one of three doses (25 mg twice a day, 100 mg three times a day, or 500 mg twice a day) or zidovudine (100 mg five times a day). Clinical and laboratory assessments were performed weekly. Viral isolates were obtained from a subgroup of patients before and after treatment and were evaluated for in vitro sensitivity to L-697,661.

Results. Both L-697,661 and zidovudine were well tolerated. Transient increases in CD4 counts were noted in the patients with fewer than 200 CD4 cells per cubic millimeter who received the two higher doses of L-697,661, but not in those who received the lowest dose or zidovudine. Patients who received L-697,661 had rapid, dose-related decreases in plasma p24 antigen levels. However, this response virtually disappeared after six weeks in some patients receiving L-697,661, coincidently with the emergence of resistant viruses. This change in susceptibility was more frequent among patients receiving the higher doses of L-697,661 and was associated with amino acid substitutions at positions 103 and 181 in the HIV-1 reverse transcriptase gene.

Conclusions. L-697,661 is safe and well tolerated and has significant dose-related activity against HIV-1. However, resistant strains of the virus emerge rapidly and may limit the effectiveness of non-nucleoside reverse transcriptase inhibitors as monotherapy for HIV-1 infection. (N Engl J Med 1993;329:1065-72.)

CURRENT treatment of human immunodeficiency virus type 1 (HIV-1) infection consists exclusively of the nucleoside-analogue reverse transcriptase inhibitors zidovudine, didanosine, and zalcitabine.[1-8] Unfortunately, the antiretroviral and clinical effectiveness of nucleoside therapy diminishes over time. It remains unclear whether the loss of effectiveness of these agents is due to incomplete inhibition of viral replication, the development of resistant viral variants, or other mechanisms of pathogenesis.[9-11] Therefore, the search for new agents that inhibit viral replication more completely, preferably through complementary mechanisms of action, has intensified.

A new group of non-nucleoside reverse transcriptase inhibitors has been developed over the past several years.[12-17] In contrast to the nucleoside analogues, which inhibit the reverse transcription process by incorporation into the elongating DNA strand with resultant chain termination, the non-nucleoside agents act through direct inhibition of reverse transcriptase and are not incorporated into the growing DNA chain. These agents, which include the tetrahydroimidazobenzo-diazepinone (TIBO) derivatives,[12] alpha-anilino phenylacetamide derivatives,[13] delavirdine (U-90152),[14] nevirapine (BI-RG 587),[15] and the pyridinone derivatives,[16,17] selectively inhibit HIV-1 (but not HIV-2) reverse transcriptase at nanomolar concentrations and are active against HIV-1 isolates that are resistant to zidovudine.[16,17]

Recently, one compound from the pyridinone group — L-697,661 — was selected for further development in phase 1–2 clinical trials. Preclinical studies and early pharmacokinetic studies in humans demonstrated that it had good oral bioavailability, with serum levels of more than 1 μmol per liter after a single oral dose of 500 mg, and an acceptable safety profile. In this paper, we describe the results of two initial clinical trials of this new compound.

METHODS

Patients

Two independent, concurrently run clinical trials were initiated in May 1991 at the University of Alabama at Birmingham. The protocol for each trial was approved by the university's institutional review board. Patients with HIV-1 infection whose CD4 counts ranged from 200 to 500 cells per cubic millimeter were treated under protocol A, and patients with counts below 200 per cubic millimeter were treated under protocol B. Both protocols specified that patients had to be more than 17 years old, not be pregnant, have normal renal and hepatic function (all laboratory values had to be less than twice the upper limit of normal), be seronegative for hepatitis B surface antigen, not be taking recreational drugs or abusing alcohol, and be able to give informed consent. Patients were excluded from treatment under protocol A if they had had evidence of the acquired immunodeficiency syndrome (AIDS) (i.e., an AIDS-defining condition). Patients were eligible for treatment under protocol B if they had previously had *Pneumocystis carinii* pneumonia or stable Kaposi's sarcoma but were ineligible if they had a history of any

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Supported by a grant from Merck Research Laboratories and by grants from the National Institutes of Health (AI-27767 and AI-27290), the General Clinical Research Center (NIH NCRR 5M01-RR00032), and the U.S. Army Medical Research Acquisition Activity (DAMD-17-90-C-0064 and DAMD-17-93-C-3146).

*The members of the L-697,661 Working Group are listed in the Appendix.

other AIDS-defining condition. Patients could be treated under either protocol if they had undergone antiretroviral therapy with zidovudine, but could not have received the drug for a minimum of 14 days before they began receiving study medication. No investigational or immunosuppressive agents could be given within 30 days before study entry.

Study Design and Treatment Regimens

The studies were double-blind, randomized, parallel-group six-week clinical trials. Under both protocols, patients were assigned to one of the following treatments according to a randomized allocation schedule: a low dose of L-697,661 (25 mg orally every 12 hours), a medium dose of L-697,661 (100 mg orally every 8 hours), a high dose of L-697,661 (500 mg orally every 12 hours), or standard therapy with zidovudine (100 mg orally every 4 hours, five times daily). Fifteen patients were sought for each of the four treatment groups in both protocols (total target enrollment, 120 patients). At the end of the six-week study period, the study treatment was stopped for a one-week washout period during which additional tests for surrogate markers of antiviral activity were performed. After this period, the patients were allowed to continue taking study medications in a double-blind fashion as part of an extension protocol. Patients in protocol B continued to receive therapy for an additional six weeks according to their originally assigned regimens. Patients in protocol A who had taken L-697,661 continued to receive it according to their original treatment assignments; however, those in protocol A who had originally taken zidovudine were randomly reassigned to receive one of the three doses of L-697,661.

Evaluation and Follow-up

During the two weeks before enrollment, all patients underwent a physical examination, electrocardiography, and a clinical laboratory evaluation, and a complete history was obtained. Base-line laboratory studies and physical examination were repeated on the day of study entry and on each subsequent visit. Patients were assessed weekly throughout the six-week study period and again during study week 7 after the one-week washout period. During the six-week extension, patients were evaluated every other week.

Antiretroviral activity was assessed by weekly measurement of CD4 cell counts and plasma HIV-1 p24 antigen levels (Abbott Laboratories, Chicago). A p24 antigen test was defined as positive if it detected more than 4 pg of p24 antigen per milliliter (a majority of patients with detectable p24 antigen had levels above 25 pg per milliliter). As part of the protocol, aliquots of plasma and peripheral-blood mononuclear cells (PBMCs) were collected periodically throughout the study and stored at −70°C and −156°C, respectively, for future use, including evaluation for the development of resistance.

Virus Isolation

Blood for isolation of the virus was obtained from all patients on the day therapy was initiated (day 0) and again on day 49, one week after therapy was discontinued. Pretreatment and post-treatment viral isolates from 22 randomly chosen patients were selected for evaluation. HIV-1 was isolated from the PBMCs by cocultivation with phytohemagglutinin-stimulated donor PBMCs as previously described.[18] Thus, all viral isolates represented primary virus amplifications.

Viral-Isolate Expansion and Sensitivity Assay

Paired primary viral isolates (pretreatment and post-treatment isolates) were thawed and added separately to 5.0×10^6 activated, uninfected normal donor PBMCs in 5.0 ml of culture medium. Additional activated PBMCs were added at two-day intervals; seven days after initiation, the final culture contained approximately 4.0×10^6 cells per milliliter in 40 ml. After two more days of incubation, the culture medium was collected, centrifuged to remove residual cells, and stored at −70°C in 1.0-ml aliquots. Viral p24 antigen in the stored medium was measured with a commercial assay (Coulter, Hialeah, Fla.).

Sensitivity assays were performed in 48-well cell-culture plates. Each well contained 5.0×10^5 activated, uninfected human PBMCs in a total volume of 0.5 ml of culture medium. The same inoculum of virus (5 to 500 pg of p24 antigen) was used to evaluate both pretreatment and post-treatment isolates. Test compounds were added to the culture wells in a twofold-dilution series. Twenty-four hours after the start of the assay, the viral inoculum was removed by harvesting and washing the cells from each well. The cells were then resuspended in fresh medium containing the appropriate concentration of test compound and were seeded in 96-well cell-culture plates. Each well received 2.5×10^5 cells in 0.25 ml. Cultures were fed with compound-containing medium, and viral p24 antigen levels were determined every two to three days.

All assays were performed in quadruplicate, and control cultures without test compound were included. The 90 percent inhibitory concentration (IC_{90}) was determined on day 11 of the assay by comparing the mean p24 antigen levels in test cultures with the levels expressed by the control cultures. The IC_{90} value was the lowest actual concentration of test compound that inhibited p24 antigen expression by at least 90 percent.

Sequencing the Reverse Transcriptase Coding Region

DNA was extracted from the cryopreserved PBMCs (approximately 1.0×10^6 to 5.0×10^6 cells) from the viral-isolate expansion. The viral reverse transcriptase gene (1680 bp) was amplified by means of a nested-primer polymerase chain reaction (PCR). The sequences of the flanking primer pair were (sense) 5′GGACCTACACCTGTCAACAT (nucleotides 2483 to 2502 of HXB2) and (antisense) 5′TCACTAGCCATTGCTCTCCA (nucleotides 4283 to 4302). The sequences of the nested primer pair were (sense) 5′CCGACCTGCATAGAATTCATGCC(C/A)ATTAGTCCTATTGA (nucleotides 2549 to 2565) and (antisense) 5′GCCCCGACCTGCATAAAGCTTATAGTAC(C/T)TTCCTGATTCC (nucleotides 4211 to 4229). The nested primers included either ATG initiation or TGA termination codons and restriction-enzyme sites to facilitate cloning and expression of reverse transcriptase. The first-round PCR reaction mixture contained PCR buffer I (Perkin–Elmer Cetus, Norwalk, Conn.), 200 μmol of each deoxynucleoside triphosphate per liter, 1 μmol of each flanking primer per liter, 0.5 μg of template DNA, and 2 units of *Taq* polymerase in a final volume of 50 μl. Amplification was carried out for one minute at 94°C, one minute at 56°C, and three minutes at 72°C, for 25 cycles. On completion, 3 μl of the reaction product was added to a second-round PCR mixture, which contained 1 μmol of each nested primer per liter in a 100-μl reaction volume. PCR was carried out for one minute at 94°C, one minute at 45°C, and three minutes at 72°C, for five cycles. The annealing temperature was then raised from 45°C to 52°C, and the samples were amplified for an additional 30 cycles. The reverse transcriptase PCR products were cloned into the *Eco*RI and *Hin*dIII sites of bacterial expression plasmid pLG18-1, a derivative of pRT1-lacI[19] in which the reverse transcriptase coding sequences were replaced with polylinker *Hin*dIII/*Eco*RI Genblock (Pharmacia LKB Biotechnology, Piscataway, N.J.). The expression plasmids were screened for the presence of a functional reverse transcriptase with use of the in situ assay, essentially as described by Prasad and Goff,[20] to eliminate the sequencing of clones containing stop codons. Reverse transcriptase expression was induced with 200 μmol of isopropyl-β-D-thiogalactopyranoside for six hours. The reverse transcriptase coding region from three molecular clones that exhibited enzymatic activity in the in situ assay was sequenced. Sequence analysis was confined to the N-terminal 343 codons of the reverse transcriptase gene.

Statistical Analysis

Differences in base-line characteristics among the treatment groups were assessed with an extension of Fisher's exact test for categorical outcomes[21] and analysis of variance for continuous outcomes (on ranks for CD4 counts and p24 antigen levels). Drug safety and tolerability were assessed by tabulating clinically relevant adverse events. Fisher's exact test was used to compare the observed incidence rates among and between the treatment groups.

Adverse events were recorded only if they began between the time of the first dose and the end of the extension period.

Mixed-effect models[22,23] were used to analyze the temporal change in each surrogate marker during the first six weeks of treatment. The analysis focused on the first six weeks because the treatment of some patients in protocol A was changed from zidovudine to L-697,661. For each patient, the logarithm of the ratio of values from week 1 through week 6 to base-line values was modeled as a simple, random slope over time. The analysis of p24 antigen values was restricted to patients whose pretreatment levels were above the detectable limit (i.e., >4 pg per milliliter); if levels were undetectable on subsequent visits, the minimal detectable level was recorded. The patients were evaluated according to their protocol, except in the evaluation of p24 antigen values; the few patients in protocol A who had detectable levels of the antigen (18 patients) were evaluated together with the patients in protocol B.

Significant results ($P<0.05$) are reported without adjustment for multiple comparisons, and all P values are based on two-sided tests.

Results

Base-Line Characteristics

A total of 68 patients in protocol A and 67 patients in protocol B were randomly assigned to the four treatment regimens. Only four patients (two in each study) were nonwhite. Most of the patients were men (93 percent), and the average age was 36 years. No significant demographic differences between the treatment groups were detected in either study. Furthermore, there were no clinically important differences between the treatment groups in the results of pretreatment laboratory tests for drug safety. As expected, the proportion of patients with detectable plasma p24 antigen levels before treatment was greater in protocol B (69 percent) than in protocol A (26 percent). Among the patients in protocol A, there were significant differences in median pretreatment CD4 counts between those receiving 50 mg of L-697,661 (395 per cubic millimeter) and those receiving 300 mg (243 per cubic millimeter, $P = 0.009$) or 1000 mg (294 per cubic millimeter, $P = 0.05$) or those receiving zidovudine (294 per cubic millimeter, $P = 0.02$). No within-protocol treatment differences were detected in the frequency of pretreatment p24 antigenemia.

Among the patients in protocols A and B, 59 (87 percent) and 55 (82 percent), respectively, had previously received zidovudine therapy; most (38 and 45, respectively) had undergone therapy for more than six months. In both protocols, a greater proportion of patients assigned to the low dose of L-697,661 had received zidovudine, but this difference was not significant.

Treatment Period

Eight patients (12 percent) in protocol A and 18 patients (27 percent) in protocol B did not complete the full 13-week study period. One other patient in protocol B was withdrawn from treatment shortly before the visit during week 6 because of elevated liver-enzyme levels; treatment was resumed after the washout period, without subsequent hepatic dysfunction. Only two patients in protocol A were withdrawn because of adverse clinical events (nausea in both patients and headache and lethargy in one each). Ten patients in protocol B were withdrawn because of adverse clinical events (esophageal ulcer and candidiasis in one patient, pulmonary cryptococcosis in one, worsening Kaposi's sarcoma requiring chemotherapy in one, desquamating rash in two, constitutional symptoms in three, cerebral toxoplasmosis in one, and peripheral neuropathy in one). Three adverse clinical events in combination with elevated serum aminotransferase levels were noted: Hodgkin's lymphoma in one patient (protocol A), rash in another (protocol A), and fever in a third (protocol B). Two patients (one in each protocol) were withdrawn during the first week of the study when they were found to have been ineligible for entry.

Safety and Tolerability

The adverse events are summarized in Table 1. There were no differences in the frequency of fever, headache, and asthenia or fatigue. However, significantly more patients receiving 300 mg of L-697,661 had rashes than those receiving zidovudine ($P = 0.04$). More patients receiving zidovudine reported at least one episode of nausea than those receiving L-697,661, but this difference was not significant. One patient in protocol A who was receiving L-697,661 (1000 mg) was temporarily withdrawn from treatment because of elevated serum liver-enzyme levels (aspartate and alanine aminotransferase); treatment was resumed with a lower dose (300 mg per day), with no recurrence of hepatic dysfunction. No patient had a significant elevation of the serum total bilirubin or serum creatinine level. Abnormally low hemoglobin levels (≤8 g per deciliter) were not observed in any patient in protocol A, but were seen in four patients in protocol B (one patient per treatment group). No patient had a white-cell count below 1000 per cubic millimeter. A serious opportunistic infection and a neo-

Table 1. Commonly Reported Adverse Events.*

Reaction	L-697,661, 50 mg (N = 34)	L-697,661, 300 mg (N = 33)	L-697,661, 1000 mg (N = 34)	Zidovudine, 500 mg (N = 34)
	no. (%) of patients with reaction			
Headache	15 (44)	11 (33)	14 (41)	10 (29)
Asthenia or fatigue	6 (18)	4 (12)	4 (12)	4 (12)
Rash	8 (24)	8 (24)†	7 (21)	2 (6)
Fever	8 (24)	7 (21)	6 (18)	6 (18)
Nausea	5 (15)	4 (12)	6 (18)	9 (26)
Vomiting	2 (6)	0	1 (3)	2 (6)
Diarrhea	14 (41)	7 (21)	10 (29)	7 (21)
Elevation of liver enzymes‡				
ALT	3 (9)	0	2 (6)	1 (3)
AST	3 (9)	1 (3)	1 (3)	0

*Adverse events were counted only through week 6 in patients in protocol A who received zidovudine; events were counted in all other groups through week 13. There were no significant differences between the treatment groups in the frequency of adverse events except for rash.

†$P = 0.04$ for the comparison with patients receiving zidovudine.

‡Levels were considered elevated if more than five times the upper limit of normal. ALT denotes alanine aminotransferase, and AST aspartate aminotransferase.

plasm developed in two patients before the conclusion of the 13 weeks of study (Hodgkin's lymphoma in a patient in protocol A receiving 50 mg of L-697,661 and cerebral toxoplasmosis in a patient in protocol B receiving 50 mg of L-697,661). One patient died during the study period, but had been withdrawn from the study for over two weeks before death (zidovudine, protocol B).

Measurements of Antiretroviral Activity

Mixed-effects–model estimates of the change from base line in the CD4 count after one week and six weeks of treatment are shown in Table 2. None of the changes were significant among the patients in protocol A. Among the patients in protocol B, significant increases from base line were seen at week 1 in those receiving 300 mg of L-697,661 (53.2 percent, $P<0.001$) and those receiving 1000 mg (29 percent, $P = 0.01$), although the CD4 counts in each of these groups returned to close to base line by week 6 (6.7 percent above base line and 2.7 percent below base line, respectively). In contrast, the patients receiving the low dose of L-697,661 (50 mg) had an immediate and progressive fall from base line in the CD4 counts; the decrease became significant by week 6 (32.1 percent, $P = 0.002$). CD4 counts did not increase significantly from base line among the patients receiving zidovudine (4.8 percent at week 1 and 4.6 percent at week 6, $P>0.05$ for both).

Mixed-effects–model estimates for the change from base line in the p24 antigen level at week 1 and week 6 are also shown in Table 2. All patients receiving L-697,661 had significant declines in p24 antigen levels from base line at week 1 (19.2 percent in those receiving 50 mg, $P = 0.04$; 31.9 percent in those receiving 300 mg, $P = 0.002$; and 42.1 percent in those receiving 1000 mg, $P<0.001$); however, only those receiving 300 mg had a significant decrease at week 6 (34.1 percent, $P = 0.015$). A trend toward decreased p24 antigen levels was observed in the patients receiving zidovudine, but this trend did not reach statistical significance. In the patients receiving 1000 mg of L-697,661, the slope estimate for p24 antigen levels between week 1 (−42.1 percent) and week 6 (−17.0 percent) was significant ($P<0.05$), thus raising the possibility that HIV-1 had become resistant to the drug.

Viral-Susceptibility Assays

Table 3 shows the IC_{90} values for L-697,661 and zidovudine in viral isolates from 19 randomly selected patients before and after treatment. All pretreatment isolates (week 0) were found to be sensitive to L-697,661; the IC_{90} ranged from 25 to 800 nmol per liter. Loss of sensitivity to L-697,661, indicated by an eightfold or greater difference between pretreatment and post-treatment isolates, was observed in all five patients receiving the high dose of the drug (1000 mg per day), four of five receiving the medium dose (300 mg), and two of six receiving the low dose (50 mg). The development of drug resistance generally coincided with increases in the plasma p24 antigen level between weeks 1 and 6 of treatment (Fig. 1). As expected, changes in sensitivity to zidovudine were not found in isolates from the patients treated with L-697,661, nor were changes in sensitivity to L-697,661 observed in isolates from patients treated with zidovudine.

The reverse transcriptase coding regions of the 19 pairs of isolates were sequenced and analyzed for amino acid substitutions known from in vitro studies to contribute to phenotypes showing resistance to L-697,661 and zidovudine.[10,11,24] Three molecular clones from each isolate representing the predominant viral genotype were sequenced. The results, summarized in Table 3, reveal a perfect correlation between

Table 2. Changes from Base Line in CD4 Counts and Plasma p24 Antigen Levels after One and Six Weeks of Treatment.*

VARIABLE	L-697,661, 50 mg	L-697,661, 300 mg	L-697,661, 1000 mg	ZIDOVUDINE, 500 mg
	estimated mean % change from base line (95% confidence interval)			
CD4 count				
Protocol A (355/64)†				
Week 1	−9.5 (−22.3 to 5.4)	6.6 (−8.0 to 23.5)	8.4 (−6.2 to 25.2)	15.7 (−0.2 to 34.2)
Week 6	−14.2 (−27.5 to 1.6)	−3.7 (−18.4 to 13.7)	−8.3 (−22.3 to 8.2)	−3.6 (−18.3 to 13.8)
Protocol B (304/60)†				
Week 1	−15.3 (−30.1 to 2.6)	53.2 (25.2 to 87.3)‡	29.0 (5.7 to 57.6)§	4.8 (−14.6 to 28.6)
Week 6	−32.1 (−46.9 to −13.2)¶	6.7 (−17.3 to 37.6)	−2.7 (−24.6 to 25.5)	4.6 (−20.4 to 37.4)
p24 Antigen				
Both protocols (328/60)†				
Week 1	−19.2 (−34.2 to −0.8)‖	−31.9 (−46.3 to −13.6)¶	−42.1 (−53.9 to −27.1)‡	−24.5 (−44.4 to 2.5)
Week 6	−20.9 (−40.6 to 5.3)	−34.1 (−53.0 to −7.8)**	−17.0 (−39.9 to 14.7)	−25.1 (−50.9 to 14.2)

*Values were calculated by mixed-effects modeling of log ratio to base line (see the Methods section). All significant changes from base line are indicated by the footnote symbols in the body of the table.

†Number of observations in model/number of patients.

‡$P<0.001$ for the comparison with base line.

§$P = 0.01$ for the comparison with base line.

¶$P = 0.002$ for the comparison with base line.

‖$P = 0.04$ for the comparison with base line.

**$P = 0.015$ for the comparison with base line.

Table 3. Resistance-Associated Amino Acid Mutations and Susceptibility of Viral Isolates.

PATIENT NO.*	L-697,661		ZIDOVUDINE	
	IC_{90}†	SUBSTITUTIONS‡	IC_{90}†	SUBSTITUTIONS‡
L-697,661				
1000 mg				
1				
Before	400	None	800	None
After	≥12,000	Y181C	400	None
2				
Before	50	None	3000	M41L,T215Y
After	≥12,000	Y181C	800	K70R
3				
Before	200	None	100	K70R
After	≥12,000	Y181C	200	K70R
4				
Before	50	None	1500	M41L,T215Y/F
After	≥12,000	Y181C	1500	M41L,T215Y
5				
Before	400	None	400	K70R
After	≥12,000	Y181C	200	K70R
300 mg				
6				
Before	100	None	50	None
After	≥12,000	Y181C	25	None
7				
Before	400	None	100	None
After	≥12,000	K103N,Y181C	50	None
8				
Before	50	None	100	None
After	≥6,000	K103N,Y181C	50	None
9				
Before	100	None	25	None
After	800	K103Q	25	None
10				
Before	100	None	12	None
After	200	None	25	None
50 mg				
11				
Before	25	None	≥3000	M41L,T215Y
After	6,000	Y181C	≥3000	M41L,T215Y
12				
Before	400	None	100	K70R
After	400	None	200	K70R
13				
Before	200	None	≥3000	M41L,T215Y
After	200	None	≥3000	M41L,T215Y/C
14				
Before	100	None	3000	M41I
After	≥12,000	Y181C	3000	M41L/T215Y
15				
Before	200	None	800	None
After	400	None	800	None
16				
Before	400	K103R	≥3000	D67N,K70R,K219Q
After	400	K103R	≥3000	D67N,K70R,K219Q
Zidovudine				
17				
Before	200	None	≥3000	M41L,D67N,T215Y
After	400	None	≥3000	M41L,D76N,T215Y
18				
Before	50	None	100	None
After	100	None	200	None
19				
Before	800	None	≥3000	M41L,D67N,T215Y
After	800	None	≥3000	M41L,D67N,T215Y

*"Before" denotes isolates obtained before treatment, and "After" isolates obtained after treatment.

†Sensitivity assays were performed as described in the Methods section. IC_{90} values are expressed in nanomoles per liter.

‡Amino acid substitutions associated with drug resistance are designated by the single-letter amino acid code (Y denotes tyrosine, C cysteine, M methionine, L leucine, K lysine, R arginine, T threonine, F phenylalanine, N asparagine, I isoleucine, D aspartic acid, and Q glutamine). The number refers to the residue position within the reverse transcriptase protein. The first letter is the amino acid residue found in wild-type, sensitive reverse transcriptase proteins. The second letter is the resistance-associated substitution identified in treated patients and known from in vitro studies to confer resistance.[10,11,24] "None" indicates that no deviations from the wild-type sequence were noted at these residues (positions 103 and 181 for L-697,661[24] and positions 41, 67, 70, 215, and 219 for zidovudine[10,11]).

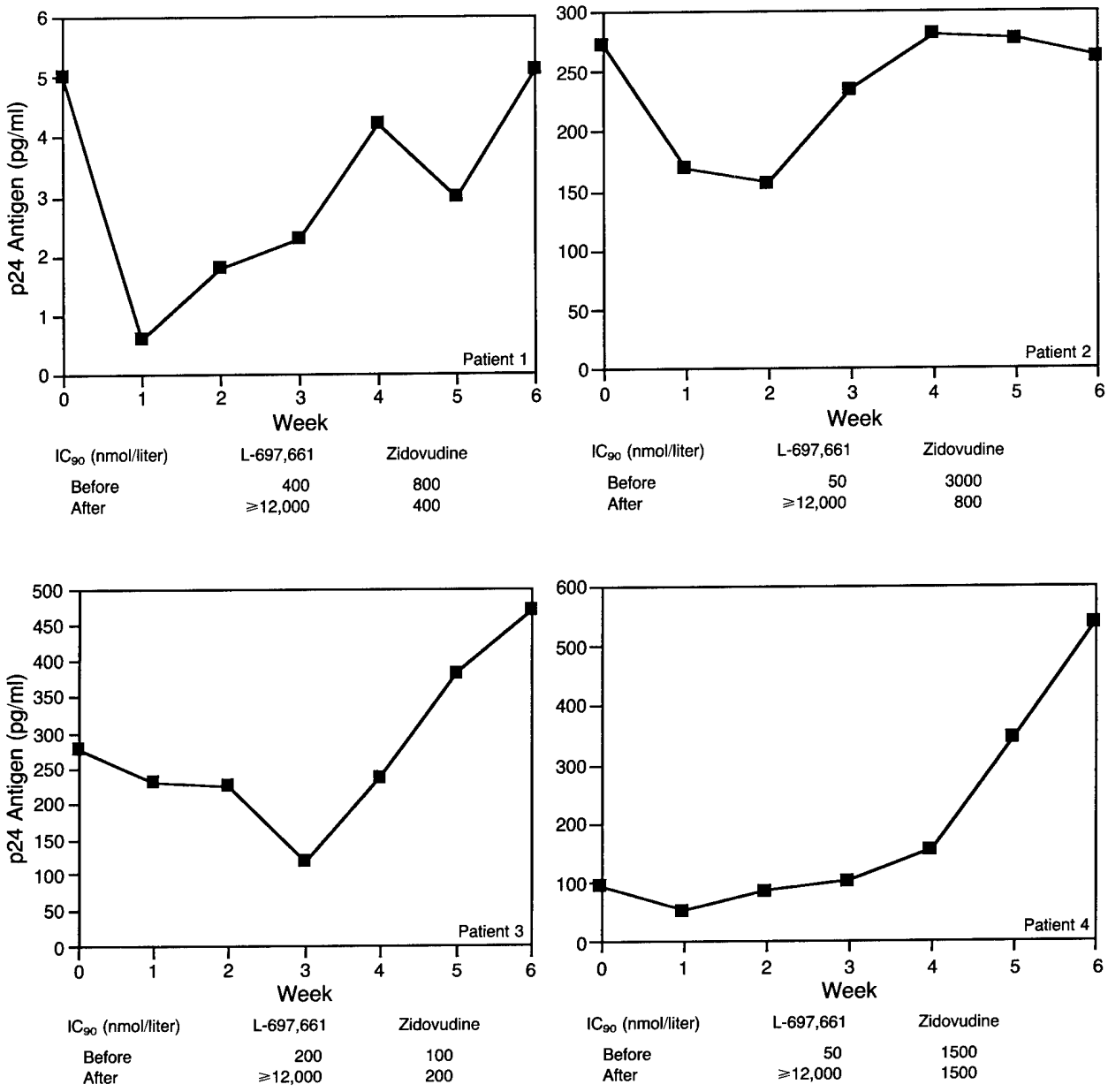
reduced viral sensitivity to the non-nucleoside inhibitor and selection for mutational alterations at reverse transcriptase residue 103 or 181 (or both). An 8-fold to 10-fold loss of sensitivity to L-697,661 was associated with a substitution of glutamine for lysine at position 103 (Patient 9). A higher level of resistance (≥30-fold loss of sensitivity) was associated exclusively with a substitution of cysteine for tyrosine at position 181, occasionally expressed with a substitution of asparagine for lysine at position 103. A substitution of arginine for lysine at position 103 in an isolate from Patient 16 was not associated with reduced drug sensitivity. Resistance to zidovudine correlated with alterations at residues 41, 67, 70, 215, and 219, as reported previously.[10,11]

The pretreatment and post-treatment viral isolates from Patients 3, 8, and 9 were tested for cross-resistance to other non-nucleoside reverse transcriptase inhibitors — L-697,229, nevirapine, and R82913 (a TIBO derivative). As compared with the pretreatment isolates, which were uniformly sensitive to these agents, the post-treatment isolates were resistant to all three inhibitors, showing increases of 15-fold to 240-fold in the IC_{90} (data not shown).

DISCUSSION

This study reports on the clinical activity and safety profile of L-697,661, a novel antiretroviral agent representative of the non-nucleoside reverse transcriptase inhibitors. All three doses of L-697,661 were well tolerated, and their safety profile was comparable to that of zidovudine. Over the 13-week study period, a small proportion of the patients were withdrawn from treatment because of adverse reactions. With few exceptions, these reactions were evenly distributed among all treatment groups and between the two protocol groups. Nausea was a common side effect but occurred more often among patients receiving zidovudine. Rash was the only adverse reaction that occurred more frequently among those receiving L-697,661. Although preclinical studies indicated the potential hepatotoxicity of L-697,661, very few of the patients receiving L-697,661 had markedly elevated serum aminotransferase levels; there was no significant difference between the patients receiving L-697,661 and those receiving zidovudine in the frequency of these abnormal values.

Previous studies of zidovudine and other nucleoside agents generally have observed a significant increase in the CD4 count over the first 6 to 8 weeks of therapy, with a return toward base line by 24 weeks.[1-3,25] In the present study, the CD4 counts of the patients receiving the medium or high dose of L-697,661 were increased significantly at week 1 but returned to base line by week 6 (Table 2); the counts of the patients receiving the low dose showed an immediate and progressive decline. The CD4 counts of the patients receiving zidovudine did not change significantly over the treatment period, probably because over 80 percent of the patients had received zidovudine therapy previously.



IC_{90} (nmol/liter)	L-697,661	Zidovudine
Before	400	800
After	≥12,000	400

IC_{90} (nmol/liter)	L-697,661	Zidovudine
Before	50	3000
After	≥12,000	800

IC_{90} (nmol/liter)	L-697,661	Zidovudine
Before	200	100
After	≥12,000	200

IC_{90} (nmol/liter)	L-697,661	Zidovudine
Before	50	1500
After	≥12,000	1500

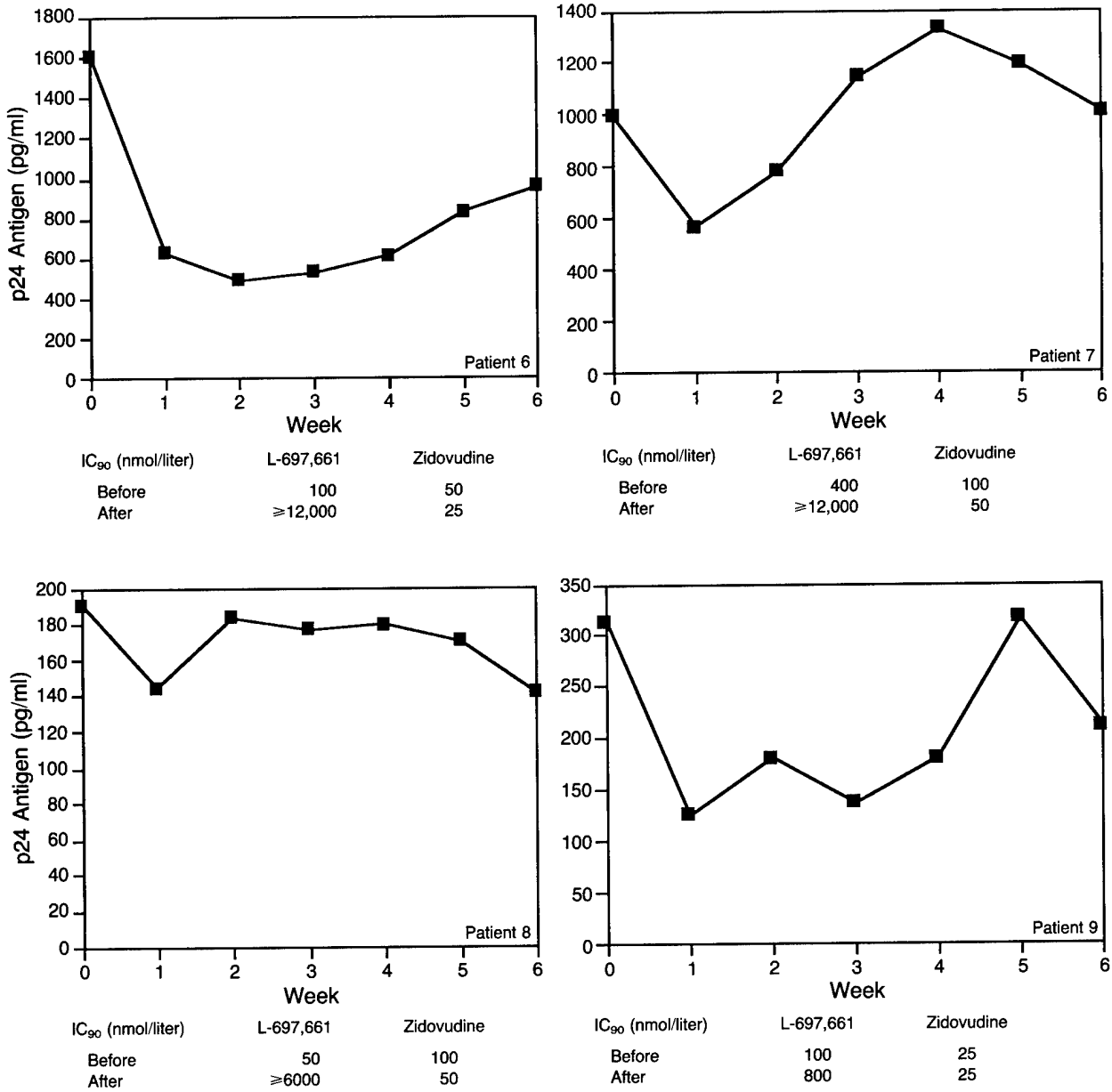
Figure 1. Plasma p24 Antigen Levels and IC_{90} Values for L-697,661 and Zidovudine in HIV-1 Isolates from Eight Representative Patients Treated with L-697,661.

Patients 1 through 4 received 500 mg every 12 hours, and Patients 6 through 9 received 100 mg every 8 hours. Plasma p24 antigen levels were measured with a commercial assay according to the manufacturer's directions. IC_{90} values denote the inhibition of matched pretreatment isolates ("Before") and post-treatment isolates ("After") by L-697,661 and zidovudine (see the Methods section).

Changes in plasma p24 antigen levels generally correlated inversely with changes in CD4 counts, with a significant early (week 1) but unsustained fall in response to L-697,661 (Table 2). In many patients, the fall in the p24 antigen level at week 1 was followed by a progressive increase to base-line or even higher values (Fig. 1), suggesting that viral drug resistance had developed.

Previous studies of HIV-1 isolates exposed to L-697,661 in vitro have demonstrated a marked reduction in viral susceptibility to the drug in association with single point mutations at either of two critical amino acid positions (103 and 181) within the reverse transcriptase gene product.[24] On the basis of these findings, we analyzed pretreatment and post-treatment isolates from a subgroup of patients for susceptibility to L-697,661 and sequenced their reverse transcriptase genes.

This analysis showed that treatment with L-697,661 resulted in the rapid selection of HIV-1 variants that were resistant to inhibition by the compound. Viral sensitivity to L-697,661 was not influenced by previous treatment with zidovudine, and conversely, changes in viral sensitivity to zidovudine were not ob-



Patient 6		
IC_{90} (nmol/liter)	L-697,661	Zidovudine
Before	100	50
After	≥12,000	25

Patient 7		
IC_{90} (nmol/liter)	L-697,661	Zidovudine
Before	400	100
After	≥12,000	50

Patient 8		
IC_{90} (nmol/liter)	L-697,661	Zidovudine
Before	50	100
After	≥6000	50

Patient 9		
IC_{90} (nmol/liter)	L-697,661	Zidovudine
Before	100	25
After	800	25

served after treatment with the non-nucleoside inhibitor. Analysis of the nucleic acid sequence of the post-treatment isolates revealed alterations at positions 103 and 181 in strains that exhibited drug resistance, but not in those that remained drug sensitive. The frequency of resistance was higher among the patients receiving the medium dose of L-697,661 (four of five patients) or the high dose (all of five patients) than in those receiving the low dose (two of six patients). The absence of the development of resistance to L-697,661 among patients receiving the low dose is most likely due to insufficient selective pressure. Taken together with the CD4 and p24 antigen responses (Table 2), the data argue for a clinically important dose-related antiviral effect of L-697,661.

The rapidity with which resistant viral populations were selected reflects a previously unsuspected dynamism of HIV-1 in vivo. Although there has been a growing appreciation that microbiologic latency of HIV-1 in vivo does not exist,[18,26-30] our study suggests an even higher rate of ongoing viral replication, and presumably continuous cellular infection, than previously appreciated. Recent studies using PCR to detect and quantify virion-associated RNA in plasma have corroborated this finding by demonstrating continuous replication of HIV-1 throughout all stages of infection.[31] Coffin has shown in studies of avian leukosis virus and by computer modeling that even a small (4.0 percent) growth advantage for a variant virus can result in complete replacement of the parental population within 40 to 50 replication cycles.[32] These findings probably explain the rapid emergence of viral resistance to L-697,661 in our study population. A heightened appreciation of viral dynamics

will be important in the design and interpretation of future clinical evaluations of antiviral compounds specific for HIV-1.

The rapid selection of viral variants with decreased sensitivity to L-697,661 will limit the clinical usefulness of this and probably other non-nucleoside reverse transcriptase inhibitors. However, in vitro studies have shown additive or synergistic activity between nucleoside and non-nucleoside antiretroviral agents.[24,33,34] Thus, these agents may still be useful in combination regimens. Because the emergence of resistant isolates occurred in this study in the setting of established infection, when the genetic complexity of the virus is extensive and subpopulations of resistant virus are more likely,[35] the use of non-nucleoside agents for very early infection[18,36-38] or post-exposure prophylaxis may be especially advantageous.

We are indebted to John Ryan, Vinod Sardana, Jon Condra, and Mark Goldman for helpful discussions; to Jane Garrison and Dolores Wilson for their assistance in the preparation of the manuscript; to the AIDS Center of the University of Alabama at Birmingham for the use of shared laboratory facilities and administrative assistance; to the staff of the core research facilities of the Birmingham Veterans Affairs Medical Center; and above all, to the study participants, many of whom traveled great distances on a weekly basis to participate in this trial.

Appendix

The following persons are members of the L-697,661 Working Group: *University of Alabama at Birmingham* — P. Chopra, J. Conway, L. DeLoach, S. Hill, S.Y. Jiang, V. Maples, and S.R. Wang; *Merck Research Laboratories* — G.B. Calandra, A. Cnaan, L. Gotlib, P. Patterson, J.C. Quintero, A. Rhodes, C.L. Schneider, D.L. Titus, C. Uncapher, and J.A. Waterbury.

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Viral dynamics in human immunodeficiency virus type 1 infection

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Reprinted from Nature, Vol. 373, January 12, 1995

Viral dynamics in human immunodeficiency virus type 1 infection

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The dynamics of HIV-1 replication *in vivo* are largely unknown yet they are critical to our understanding of disease pathogenesis. Experimental drugs that are potent inhibitors of viral replication can be used to show that the composite lifespan of plasma virus and virus-producing cells is remarkably short (half-life ~2 days). Almost complete replacement of wild-type virus in plasma by drug-resistant variants occurs after fourteen days, indicating that HIV-1 viraemia is sustained primarily by a dynamic process involving continuous rounds of *de novo* virus infection and replication and rapid cell turnover.

THE natural history and pathogenesis of human immunodeficiency virus type-1 (HIV-1) infection are linked closely to the replication of virus *in vivo*[1-17]. Clinical stage is significantly associated with all measures of virus load, including infectious virus titres in blood, viral antigen levels in serum, and viral nucleic acid content of lymphoreticular tissues, peripheral blood mononuclear cells (PBMCs) and plasma (reviewed in ref. 18). Moreover, HIV-1 replication occurs preferentially and continuously in lymphoreticular tissues (lymph node, spleen, gut-associated lymphoid cells, and macrophages)[11,19,20]; virus is detectable in the plasma of virtually all patients regardless of clinical stage[6,10,13,21]; and changes in plasma viral RNA levels predict the clinical benefit of antiretroviral therapy (R. Coombs, unpublished results). These findings emphasize the central role of viral replication in disease pathogenesis.

Despite the obvious importance of viral replication in HIV-1 disease, relatively little quantitative information is available regarding the kinetics of virus production and clearance *in vivo*, the rapidity of virus and $CD4^+$ cell population turnover, and the fixation rates of biologically relevant viral mutations[22,23]. This circumstance is largely due to the fact that previously available antiretroviral agents lacked sufficient potency to abrogate HIV-1 replication, and methods to quantify virus and determine its genetic complexity were not sufficiently sensitive or accurate. We overcame these obstacles by treating subjects with new investigational agents which potently inhibit the HIV-1 reverse transcriptase (nevirapine, NVP)[24] and protease (ABT-538; L-735,524)[25,26]; by measuring viral load changes using sensitive new quantitative assays for plasma virus RNA[6,18,27]; and by quantifying changes in viral genotype and phenotype in uncultured plasma and PBMCs using automated DNA sequencing[28] and an *in situ* assay of RT function[29,30].

Virus production and clearance

Twenty-two HIV-1-infected subjects with $CD4^+$ lymphocyte counts between 18 and 251 per mm^3 (mean ± s.d., 102 ± 75 cells per mm^3) were treated with ABT-538 ($n=10$), L-735,524 ($n=8$) or NVP ($n=4$) as part of phase I/IIA clinical studies. The design and clinical findings of those trials will be reported elsewhere (K. Squires *et al.*, and V.A.J. *et al.*, manuscripts in preparation). Plasma viral RNA levels in the 22 subjects at baseline ranged from $10^{4.6}$ to $10^{7.2}$ molecules per ml (geometric mean of $10^{5.5}$) and exhibited maximum declines generally within 2 to 4 weeks of initiating drug therapy (Figs 1 and 2*a*). For ABT-538- and L-735,524-treated patients, virus titres fell by as much as $10^{3.9}$-fold (mean decrease of $10^{1.9}$-fold) whereas for NVP-treated patients virus fell by as much as $10^{2.0}$-fold (mean decrease of $10^{1.6}$-fold). The overall kinetics of virus decline during the initial weeks of therapy with all three agents corresponded to an exponential decay process (Figs 1 and 2*a*).

The antiretroviral agents used in this study, despite their differing mechanisms of action, have a similar overall biological effect in that they block *de novo* infection of cells. Thus the rate of elimination of plasma virus that we measured following the initiation of therapy is actually determined by two factors: the clearance rate of plasma virus *per se* and the elimination (or suppression) rate of pre-existing, virus-producing cells. To a good approximation, we can assume that virus-producing cells decline exponentially according to $y(t)=y(0)e^{-\alpha t}$, where $y(t)$ denotes the concentration of virus-producing cells at time t after the initiation of treatment and α is the rate constant for the exponential decline. Similarily, we assume that free virus $v(t)$ is generated by virus-producing cells at the rate $ky(t)$ and declines exponentially with rate constant u. Thus, for the overall decline of free virus, we obtain $v(t)=v(0)[ue^{-\alpha t}-\alpha e^{-ut}]/(u-\alpha)$. The kinetics are largely determined by the slower of the two decay processes. As we have data only for the decline of free virus, and not for virus-producing cells, we cannot determine which of the two decay processes is rate-limiting. However, the half-life ($t_{1/2}$) of neither process can exceed that of the two combined. With these considerations in mind, we estimated the elimination rate of plasma virus and of virus-producing cells by three different methods: (1) first-order kinetic analysis of that segment of the viral elimination curve corresponding to the most rapid decline in plasma virus, generally somewhere between days 3 and 14; (2) fitting of a simple exponential decay curve to all viral RNA determinations between day 0 and the nadir or inflection point (Fig. 1); and (3) fitting of a compound decay curve

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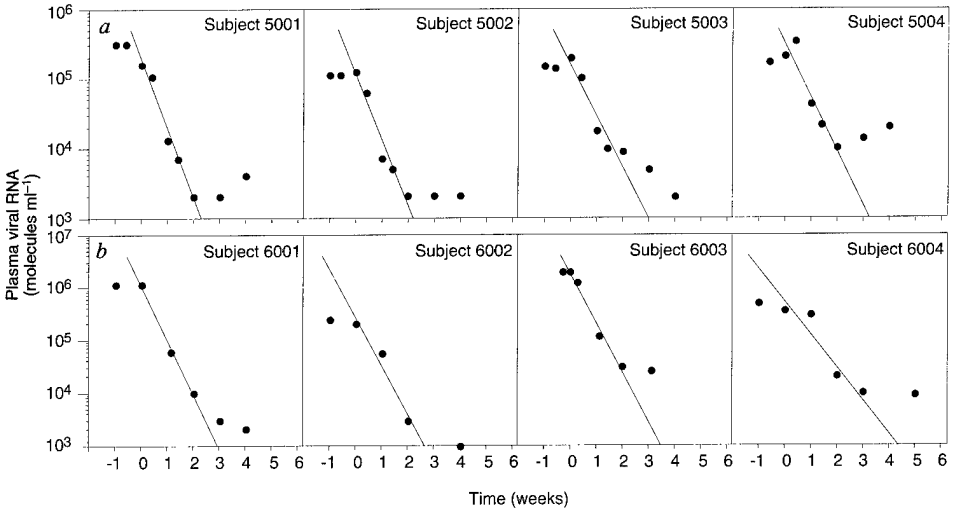


FIG. 1 Plasma viral RNA determinations in representative subjects treated with the HIV-1 protease inhibitors ABT-538 (*a*) and L-735,524 (*b*). Subjects had not received other antiretroviral agents for at least 4 weeks before therapy. Treatment was initiated at week 0 with 400–1,200 mg d^{-1} of ABT-538 or 1,600–2,400 mg d^{-1} of L-735,524 and was continued throughout the study. Viral RNA was determined by modified branched DNA (bDNA)[18] (*a*) or RT-PCR[27] (*b*) assay and confirmed by QC-PCR[6]. Shown are the least-squares fit linear-regression curves for data points between days 0 and 14 indicating exponential (first-order) viral elimination.

that takes into account the two separate processes of elimination of free virus and virus-producing cells, as described. Method (1) gives a $t_{1/2}$ of 1.8 ± 0.9 days; method (2) gives a $t_{1/2}$ of 3.0 ± 1.7 days; and method (3) gives a $t_{1/2}$ of 2.0 ± 0.9 days for the slower of the two decay processes and a very similar value, 1.5 ± 0.5 days, for the faster one. These are averages (± 1 s.d.) for all 22 patients. Method (3) arguably provides the most complete assessment of the data, whereas method (2) provides a simpler interpretation (but slightly slower estimate) for virus decline because it fails to distinguish the initial delay in onset of antiviral activity due to the drug accumulation phase, and the time required for very recently infected cells to initiate virus expression, from the subsequent phase of exponential virus decline. There were no significant differences in the viral clearance rates in subjects treated with ABT-538, L-735,524 or NVP, and there was also no correlation between the rate of virus clearance from plasma and either baseline $CD4^+$ lymphocyte count or baseline viral RNA level.

Virus turnover

Direct population sequencing. As an independent approach for determining virus turnover and clearance of infected cells, we quantified serial changes in viral genotype and phenotype with respect to drug resistance in the plasma and PBMCs of four subjects treated with NVP (Fig. 2). NVP potently inhibits HIV-1 replication but selects for one or more codon substitutions in the reverse transcriptase (RT) gene[24,31,32]. These mutations result in dramatic decreases (up to 1,000-fold) in drug susceptibility and are associated with a corresponding loss of viral suppression *in vivo*[32]. Genetic changes resulting in NVP resistance can thus serve as a quantifiable molecular marker of virus turnover. A rapid decline in plasma viral RNA was

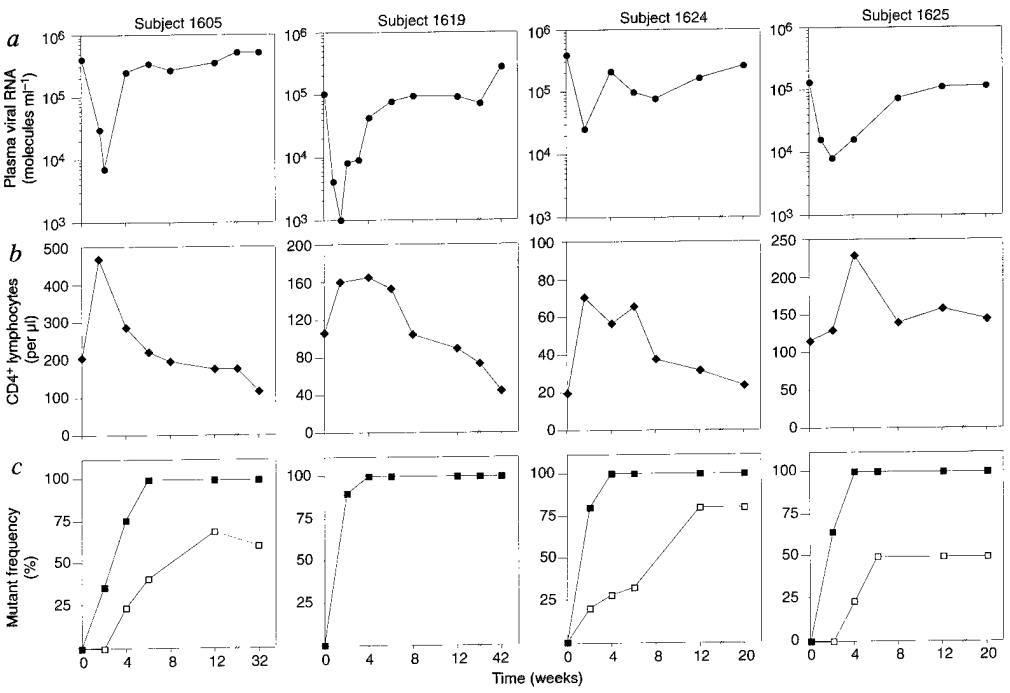


FIG. 2 Plasma viral RNA determinations (*a*), $CD4^+$ lymphocyte counts (*b*), and percentages of mutant viral genomes in plasma and PBMCs (c) of subjects initiating treatment with NVP. Subjects were participants in a clinical protocol assessing the effects of NVP when added to existing treatment with ddI (subject 1605) or ddI plus zidovudine (subjects 1619, 1624, 1625). Treatment with NVP was initiated at week 0 using 200 mg per day and was increased to 400 mg per day after 2 weeks. ddI and zidovudine dosages were 400 mg per day and 300–600 mg per day, respectively. Viral RNA (●) was determined by QC-PCR assay[6]. $CD4^+$ lymphocytes (◆) were quantified by flow cytometry. Frequencies of viral genomes containing NVP-resistance-associated mutations in plasma (■) and PBMCs (□) were determined by automated DNA sequence analysis (Fig. 3, legend), with each data point representing the average of 3–6 independent PCR amplifications and sequence determinations.

observed following the institution of NVP therapy and this was associated with a reciprocal increase in $CD4^+$ lymphocyte counts (Fig. 2*a* and *b*). Both responses were of limited duration, returning to baseline within 6–20 weeks in these four patients. The proportion of virus in uncultured plasma and PBMCs that contain NVP-resistance-conferring mutations (Fig. 2*c*) was determined by direct automated nucleotide sequencing of viral nucleic acid (Fig. 3), as previously described[28]. We first validated this method by reconstitution experiments, confirming its sensitivity for detecting RT mutants that comprise as little as 10% of the overall virus population. Defined mixtures of wild-type and mutant HIV-1 RT cDNA clones (differing only at the second base position of codon 190) were amplified and sequenced (Fig. 3*a*). Varying proportions of wild-type and mutant viral sequences present in the original DNA mixtures (mutant composition: 0, 10, 25, 50, 75 and 100%) were faithfully represented in the relative peak-on-peak heights (and in the relative peak-on-peak areas) of cytosine (C) and guanine (G) residues at the second base position within this codon. Ratios of (mutant)/(mutant + wild type) nucleotide peak heights expressed in arbitrary fluorescence units were as follows (predicted/observed): 0/< 10%; 10/18%; 25/29%; 50/49%; 75/71% and 100/94%.

We next determined the ability of direct population sequencing to quantify wild-type and mutant viral RNA genomes in clinical specimens. Figure 3*b* shows the sequence chromatograms of RT codons 179–191 from virions pelleted directly from uncultured plasma specimens of subject 1625 before (day −7) and after (days +28 and +140) the initiation of NVP therapy. At day −7, all codons within the amino-terminal half of the RT gene (codons 1–250), including those shown, were wild-type at positions associated with NVP resistance[31,32]. However, after only 28 days of NVP therapy, the wild-type plasma virus population was completely replaced by a NVP-resistant mutant population differing from the wild-type at codon 190 (glycine-to-serine substitution). After 140 days of drug therapy, this codon had evolved further such that the plasma virus population consisted of an equal mixture of two drug-resistant strains, one containing G190S and the other containing G190A. There were no other NVP-resistance-conferring mutations detectable within the viral RT gene.

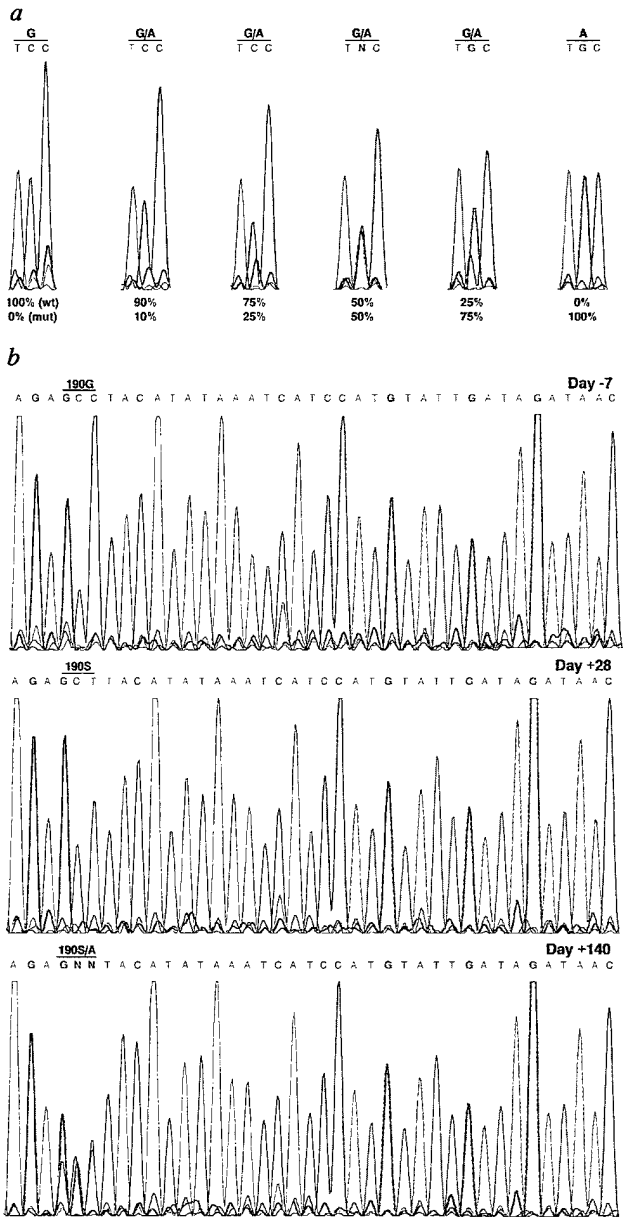
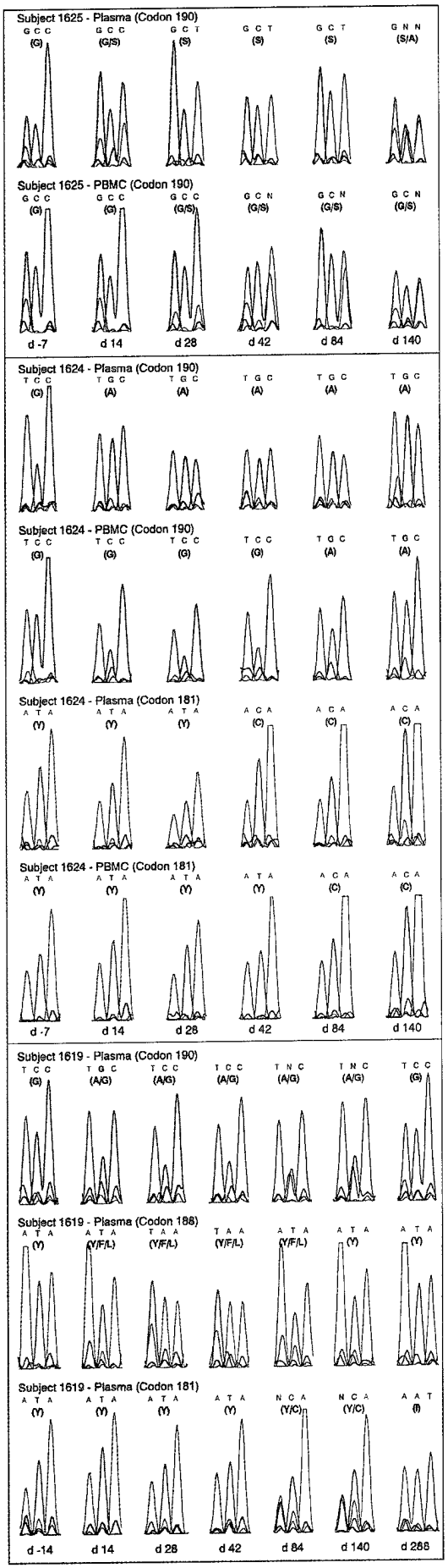


FIG. 3 Quantitative detection of HIV-1 drug-resistance mutations by automated DNA sequencing. *a*, DNA sequence chromatograms of RT codon 190 from a defined mixture of wild-type (wt) and mutant (mut) HIV-1 cDNA clones differing only at the second base position of the codon. Sequences shown were obtained from, and therefore are presented as, the minus (non-coding) DNA strand. For example, the minus-strand TCC sequence shown corresponds to the plus-strand codon GGA (glycine, G). Similarly, the minus-strand TGC sequence corresponds to the plus-strand codon GCA (alanine, A). The single-letter amino-acid code corresponds to the plus-strand DNA sequence. Mixed bases approximating a 50/50 ratio are denoted as N. *b*, DNA sequence chromatograms of RT codons 179–191 (again displayed as the minus-strand sequence) derived from plasma-virion-associated RNA of subject 1625 before (day −7) and after (days +28 and +140) starting NVP therapy. Codon changes resulting in amino-acid substitutions at position 190 are indicated for the plus strand. For example, the GCC minus-strand sequence at position 190 (day −7) corresponds to GGC (glycine, G), and the GCT minus-strand sequence at position 190 (day +28) corresponds to AGC (serine, S) in the respective plus strands.

METHODS. Mixtures of wild-type and mutant cDNA clones (*a*) were prepared and diluted such that first-round PCR amplifications were done with 1,000 viral cDNA target molecules per reaction. HIV-1 RNA was isolated from virions pelleted from uncultured plasma specimens (*b*), as described[18]. cDNA was prepared using Moloney murine leukaemia virus reverse transcriptase (GIBCO BRL)[6] and an oligonucleotide primer corresponding to nucleotides 4,283 to 4,302 of the HXB2 sequence[43]. The full-length viral reverse transcriptase gene (1,680 bp) was amplified by means of a nested PCR using conditions and oligonucleotide primers (outer primers: nt 2,483–2,502 and 4,283–4,302; inner primers: nt 2,549–2,565 and 4,211–4,229), previously reported[30]. Subgenomic fragments of the RT gene were also amplified using combinations of the following oligonucleotide primers: (5′) 2,585–2,610; (5′) 2,712–2,733; (3′) 2,822–2,844; (3′) 3,005–3,028; (3′) 3,206–3,228; (3′) 3,299–3,324; (3′) 3,331–3,350; (3′) 3,552–3,572; and (3′) 3,904–3,921. All 3′ primers incorporated the universal primer sequence for subsequent dye–primer sequence analysis. The HIV-1 copy number in every PCR reaction was determined (100–10,000 copies). A total of three to six separate PCR amplifications of primary patient material was done on each sample using different combinations of primers, and representative chromatograms are shown. Rarely, codon interpretation was ambiguous. In the day +140 plasma sample from subject 1625 (bottom of panel *b*), the complementary (plus) strand could read: AGC(serine), GCN(alanine), ACN(threonine), AGA/AGG(arginine) or GGN(glycine). In this case, we sequenced 7 full-length RT molecular clones and found that they encoded only serine or alanine. For sequencing, an automated ABI 373A sequenator and the Taq Dye Primer Cycle Sequencing Kit (ABI) were used. Sequences were analysed using Sequencher (Gene Codes Corp.) and Microgenie (Beckman) software packages, and base-pair mixtures were quantified by measuring relative peak-on-peak heights[28].



In all four subjects evaluated by direct viral population sequencing (Fig. 4), specific NVP-resistance-conferring mutations within the RT gene could be unambiguously identified and subsequently confirmed by molecular cloning, expression and drug susceptibility testing. In all cases, mutant virus increased rapidly in the plasma and virtually replaced wild-type virus after only 2–4 weeks of NVP therapy (Fig. 2*c*). By analysing the rate of accumulation of resistant mutants in the plasma population, we could obtain an independent estimate of the turnover rate of free virus. The rise of drug-resistant mutant virus is influenced substantially by the preceding increase in the $CD4^+$ cell population (which provides additional resources for virus production[33]) and therefore follows complex dynamics. However, we could obtain an estimate of these dynamics by making simplifying assumptions. We assume that wild-type virus declines exponentially with a decay rate α, and that the drug-resistant mutant increases exponentially with the rate β. Thus, the ratio of mutant to wild-type virus increases exponentially at the combined rate $\alpha+\beta$. Our genetic RNA (cDNA) data allow us to estimate this sum. Knowing α from our data on virus decline, we get $\beta \approx 0.27$, or a 32% daily virus production (average over 4 patients). Assuming that mutant virus rises exponentially, this corresponds to a doubling time of ~2 days, which is in excellent agreement with the measured elimination half-life of 2.0 ± 0.9 days for plasma virus (Figs 1 and 2*a*). Turnover of viral DNA from wild-type to drug-resistant mutant in PBMCs was delayed and less complete compared to plasma virus, reaching levels of only 50–80% of the total PBMC-associated viral DNA population by week 20 (Fig. 2*c*). Measurement of the time required for resistant virus to spread in the PBMC population allowed us also to estimate the half-life of infected PBMCs. After complete turnover of mutant virus in the plasma pool, we may assume that PBMCs infected with wild-type virus decline exponentially at a rate d, whereas cells infected by mutant virus are generated at a constant rate, but also decline exponentially at rate d. With these simplifying assumptions, the rate at which the frequency of resistant virus in the PBMC population increases provides an estimate for the parameter d and hence for the half-life of infected PBMCs. We obtained a half-life of ~50–100 days. This means that the average half-life of infected PBMCs is very long and of the same order of magnitude as the half-life of uninfected PBMCs[34,35]. Based on the long half-life of PBMCs, and the fact that these cells harbour predominantly wild-type virus at a time (days 14–28) when most virus in plasma is mutant, we conclude that most PBMCs contribute comparatively little to plasma virus load. Instead, other cell populations, most probably in the lymphoreticular system[11,19,20], must be the major source of virus production.

Direct sequence analysis of viral nucleic acid revealed not only rapid initial turnover in viral populations but also continuing viral evolution with respect to drug resistance mutations. In subject 1625 (Fig. 4, top panel), wild-type virus in plasma was completely replaced after 28 days of NVP therapy by mutant virus

FIG. 4 Quantitative detection of HIV-1 drug resistance mutations by automated DNA sequencing in plasma viral RNA (cDNA) and PBMC-associated viral DNA populations before and after the initiation of NVP on day 0. As in Fig. 3, minus-strand sequences are shown together with single-letter amino-acid codes of the corresponding plus-strand sequence. Mixed bases approximating a 50/50 ratio are denoted as N.

METHODS. HIV-1 cDNA was prepared from virions pelleted from uncultured plasma as described for Fig. 3. Viral DNA was isolated from uncultured PBMCs, as described[44]. The full-length viral reverse transcriptase genes as well as subgenomic fragments were amplified and sequenced as described for Fig. 3. The HIV-1 copy number in every PCR reaction was determined (100–10,000 copies). Some sequences were determined from both coding and non-coding DNA strands to ensure the accuracy of quantitative measurements.

TABLE 1 *In situ* functional analysis of HIV-1 RT clones

Subject	Specimen		Functional clones	NVP-sensitive clones		NVP-resistant clones	
1625	Plasma	day −7	80	80	(100%)	0	(0%)
		+14	72	27	(38%)	45	(62%)
		+28	57	0	(0%)	57	(100%)
		+84	67	0	(0%)	67	(100%)
		+140	86	0	(0%)	86	(100%)
1625	PBMC	−7	163	163	(100%)	0	(0%)
		+14	121	121	(100%)	0	(0%)
		+28	258	134	(52%)	124	(48%)
		+84	133	43	(32%)	90	(68%)
		+140	261	65	(25%)	196	(75%)
1624	Plasma	−7	19	19	(100%)	0	(0%)
		+14	34	4	(12%)	30	(88%)
		+28	79	6	(8%)	73	(92%)
		+140	27	0	(0%)	27	(100%)
1624	PBMC	−7	24	24	(100%)	0	(0%)
		+14	34	29	(85%)	5	(15%)
		+28	52	42	(81%)	10	(19%)
		+140	87	26	(30%)	61	(70%)
1605	PBMC	−7	31	31	(100%)	0	(0%)
		+140	31	11	(35%)	20	(65%)
1619	Plasma	−14	79	79	(100%)	0	(0%)
		+28	41	0	(0%)	41	(100%)
		+140	38	0	(0%)	38	(100%)

Full-length RT genes were amplified by PCR from uncultured plasma and uncultured PBMCs as described in Fig. 3 legend. DNA products were cloned into the *Eco*RI and *Hin*dIII sites of the bacterial expression plasmid pLG18-1 (refs 29, 30). The expression plasmids were screened for the presence of functional RT and tested *in situ* for susceptibility to NVP inhibition at 3,000 nM (~50–75 fold greater than the IC_{50})[24,31,32]. To ensure accuracy in distinguishing RT genes encoding NVP-resistant versus sensitive enzymes, and to confirm the identification of specific NVP-resistance-conferring RT mutations obtained by direct sequencing (Figs 3 and 4), we determined the complete nucleotide sequences of 21 cloned RT genes which had been phenotyped in the *in situ* assay (V.A.J. and G.M.S., submitted). There was complete concordance between the phenotypes and genotypes of these 21 clones with respect to NVP-resistance-conferring mutations, as well as complete concordance between direct viral population sequences and clone-derived sequences at NVP-resistance-conferring codons.

(G190S), which in turn evolved by day 140 into a mixture of G190S and G190A. In subject 1624 (Fig. 4, middle panel), two codon changes conferring NVP resistance occurred. A G190A substitution appeared in plasma virus at day 14 and a Y181C appeared at day 42. Similarly, in subject 1605 (not shown), a Y181C mutation appeared in plasma at day 14 and a Y188L mutation at day 28. The sequential changes in plasma virus were mirrored by similar changes in PBMCs at later timepoints. In subject 1619, the pattern of resistance changes was even more complex (Fig. 4, bottom panel). By day 14, approximately 70% of plasma virus contained a G190A mutation. By day +28, this mutant population was largely replaced by virus containing a Y188F/L substitution. By day 84, still another major shift in the viral quasispecies occurred, this time resulting in a population of viruses containing mutations at both Y181C and G190A. Finally, by day 288 the viral population in plasma consisted exclusively of a mutant exhibiting a single tyrosine-to-isoleucine substitution at position 181 (Y181I); mutations at codons 188 and 190 were not present in this virus population. All of these amino-acid substitutions at RT codons 181, 188 and 190 were shown in our *in situ* expression studies and by others[31,32,36] to confer high-level NVP resistance. The direct sequence analyses thus demonstrate that major changes in the HIV-1 quasispecies occur quickly and continuously in response to selection pressures and that these changes are reflected first and most prominently in the plasma virus compartment.

***In situ* RT gene expression and drug susceptibility testing.** Because direct sequence analysis of viral mixtures provides only semiquantitative information and does not distinguish between viruses with functional rather than defective RT genes, we employed another method for quantifying virus turnover in uncultured plasma and PBMC compartments. Full-length RT genes were amplified by polymerase chain reaction (PCR), cloned into pLG18-1, expressed in *Escherichia coli*, and tested individually for enzymatic function and NVP susceptibility by *in situ* assay[29,30] (Table 1). For subject 1625 at day −7, 100% (80/80) of RT clones from plasma and 100% (163/163) of RT clones from PBMCs expressed enzyme that was sensitive to NVP inhibition. By day 14, however, 62% of plasma-derived clones expressed enzyme that was resistant to NVP, and by days 28, 84 and 140, 100% were resistant. Conversely, at day 14, 0% of PBMC-derived clones expressed NVP-resistant enzyme, and even after 28, 84 and 140 days, only 48–75% of clones were resistant. Similar results were obtained for the other study subjects (Table 1). Thus, the kinetics of virus population turnover determined by a quantitative RT *in situ* expression assay corresponded closely with those determined by direct population sequencing (Fig. 2*c*).

Infectious virus drug susceptibility testing. Plasma and PBMCs are known to harbour substantial proportions of defective or otherwise non-infectious virus[6,37]. To determine whether the viral genomes represented in total viral nucleic acid (Fig. 4 and Table 1) corresponded to infectious virus with respect to NVP-resistance-conferring mutations, we co-cultivated PBMCs from three of the study subjects (1605, 1624, 1625) with normal donor lymphoblasts in order to establish primary virus isolates. The RT genes of these cultured viruses, obtained before and after therapy, were cloned (Fig. 3 and Table 1 legends) and sequenced in their entirety (V.A.J. and G.M.S., submitted). RT codons associated with NVP susceptibility were completely concordant in cultured and uncultured virus strains. Furthermore, the virus isolates exhibited NVP susceptibility profiles[38] consistent with their genotypes.

$CD4^+$ lymphocyte dynamics

Changes in $CD4^+$ lymphocyte counts during the first 28 days of therapy could be assessed in 17 of our patients (Fig. 2*b* and data not shown). $CD4^+$ cell numbers increased in every patient by between 41 and 830 cells per mm^3. For the entire group, the average increase was 186 ± 199 cells per mm^3 (mean ± s.d.), or 268 ± 319% from baseline. As $CD4^+$ lymphocytes increase in numbers because of (1) exponential proliferation of $CD4^+$ cells in peripheral tissue compartments, and/or (2) constant (linear) production of $CD4^+$ cells from a pool of precursors, we analysed our data based on each of these assumptions. The average percentage increase in cell number per day (assumption (1)) was 5.0 ± 3.1% (mean ± 1 s.d.). The average absolute increase in cell number per day (assumption (2)) was 8.0 ± 7.8 cells $mm^{-3}\ d^{-1}$. Given that peripheral blood contains only 2% of the total body lymphocytes[35] and that the average total blood volume is ~5 litres, an increase of 8 cells $mm^{-3}\ d^{-1}$ implies an overall steady-state $CD4^+$ cell turnover rate (where increases equal losses) of $(50) \times (5 \times 10^6\ mm^3) \times (8\ cells\ mm^{-3}\ d^{-1})$, or 2×10^9 $CD4^+$ cells produced and destroyed each day.

Discussion

Previously, it was shown that lymphoreticular tissues serve as the primary reservoir and site of replication for HIV-1 (refs 11, 19, 20) and that virtually all HIV-1-infected individuals, regardless of clinical stage, exhibit persistent plasma viraemia in the range of 10^2 to 10^7 virions per ml[6]. However, the dynamic contributions of virus production and clearance, and of $CD4^+$ cell infection and turnover, to the clinical 'steady-state' were obscure, although not unanticipated[22,23,39]. We show by virus quantitation and mutation fixation rates that the composite lifespan of plasma virus and of virus-producing cells is remarkably short ($t_{1/2} = 2.0 \pm 0.9$ days). This holds true for patients with $CD4^+$ lymphocyte counts as low as 18 cells per mm^3 and as high as 355 cells per mm^3 (Figs 1 and 2; G.M.S., unpublished). These findings were made in patients treated with three different antiretroviral agents having two entirely different mechanisms of action and using three different experimental approaches for assessing virus turnover. The viral kinetics thus cannot be

explained by a unique or unforeseen drug effect or a peculiarity of any particular virological assay method. Moreover, when new cycles of infection are interrupted by potent antiretroviral therapy, plasma virus levels fall abruptly by an average of 99%, and in some cases by as much as 99.99% (10,000-fold). This result indicates that the vast majority of circulating plasma virus derives from continuous rounds of *de novo* virus infection, replication and cell turnover, and not from cells that produce virus chronically or are latently infected and become activated. The identity and location of this actively replicating cell population is not known, but appears not to reside in the PBMC pool, consistent with prior reports[11,19,20]. Nevertheless, PBMCs traffic through secondary lymphoid organs and to some extent are in equilibrium with these cells[35]. It is thus possible that a small fraction of PBMCs[8,9,14–17], like a small fraction of activated lymphoreticular cells[20], could make an important contribution to viraemia.

The magnitude of ongoing virus infection and production required to sustain steady-state levels of viraemia is extraordinary: based on a virus $t_{1/2}$ of 2.0 days and first-order clearance kinetics ($v(t) = v(0)e^{-\alpha t}$, where $\alpha = 0.693/t_{1/2}$), 30% or more of the total virus population in plasma must be replenished daily. For a typical HIV-1-infected individual with a plasma virus titre equalling the pretreatment geometric mean in this study ($10^{5.5}$ RNA molecules per ml/2 RNA molecules per virion = $10^{5.2}$ virions per ml) and a plasma volume of 3 litres, this amounts to $(0.30) \times (10^{5.2}) \times (3 \times 10^3) = 1.1 \times 10^8$ virions per day (range for all 22 subjects, 2×10^7 to 7×10^9). Even this may be a substantial underestimate of virus expression because virions may be inefficiently transported from the interstitial extravascular spaces into the plasma compartment and viral protein expression alone (short of mature particle formation) may result in cytopathy or immune-mediated destruction. Because the half-life of cells producing the majority of plasma virus cannot exceed 2.0 days, at least 30% of these cells must also be replaced daily. In our patients, we estimated the rate of $CD4^+$ lymphocyte turnover to be, on average, 2×10^9 cells per day, or about 5% of the total $CD4^+$ lymphocyte population, depending on clinical stage. This rapid and ongoing recruitment of $CD4^+$ cells into a short-lived virus-expressing pool probably explains the abrupt increase in $CD4^+$ lymphocyte numbers that is observed immediately following the initiation of potent antiretroviral therapy, and suggests the possibility of successful immunological reconstitution even in late-stage disease if effective control of viral replication can be sustained.

The kinetics of virus and $CD4^+$ lymphocyte production and clearance reported here have a number of biological and clinical implications. First, they are indicative of a dynamic process involving continuous rounds of *de novo* virus infection, replication and rapid cell turnover that probably represents a primary driving force underlying HIV-1 pathogenesis. Second, the demonstration of rapid and virtually complete replacement of wild-type virus by drug-resistant virus in plasma after only 14–28 days of drug therapy is a striking example of the capacity of the virus for biologically relevant change. In particular, this implies that HIV-1 must have enormous potential to evolve in response to selection pressures as exerted by the immune system[39]. Although other studies[40–42] have provided some evidence that virus turnover occurs sooner in plasma than in PBMCs, our data show this phenomenon most clearly. A similar experimental approach involving the genotypic and phenotypic analysis of plasma virus could be helpful in identifying viral mutations and selection pressures involved in resistance to other drugs, immune surveillance and viral pathogenicity. Third, the difference in lifespan between virus-producing cells and latently infected cells (PBMCs) suggests that virus expression *per se* is directly involved in $CD4^+$ cell destruction. The data do not suggest an 'innocent bystander' mechanism of cell killing whereby uninfected or latently infected cells are indirectly targeted for destruction by adsorption of viral proteins or by autoimmune reactivities.

Although we have emphasized that most virus in plasma derives from an actively replicating short-lived population of cells, latently infected cells that become activated or chronically producing cells that generate proportionately less virus (and thus do not contribute substantially to the plasma virus pool) may nonetheless be important in HIV-1 pathogenesis. Based on *in situ* analysis[20], these cells far outnumber the actively replicating pool and the diversity of their constituent viral genomes represents a potentially important source of clinically relevant variants, including those conferring drug resistance. In future studies, it will be important not only to discern the specific elimination rates of free virus and of the most actively producing cells, but also the dynamics of virus replication and cell turnover in other cell populations and in patients at earlier stages of infection. Such information will be essential to developing a better understanding of HIV-1 pathogenesis and a more rational approach to therapeutic intervention. □

Received 22 November; accepted 16 December 1994.

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ACKNOWLEDGEMENTS. We thank the study participants; K. Squires, J. M. Kilby, M. Trechsel, L. DeLoach and the UAB 1917 Clinic staff; Abbott Laboratories, Merck & Co. and Boehringer Ingelheim Pharmaceuticals Inc. (BIPI); J. Coffin, R. May and F. Gao for discussion; J. Decker, S. Campbell-Hill, Y. Niu and S. Yin Jiang for technical assistance; and J. Wilson for artwork. This study was supported by the NIH, the US Army Medical Research Acquisition Activity, BIPI, the Wellcome Trust, Keble College and Boehringer Ingelheim Stiftung. Core research facilities were provided by the UAB Center for AIDS Research, the UAB AIDS Clinical Trials Unit and the Birmingham Veterans Administration Medical Center.

Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology
9:305–310 © 1995 Raven Press, Ltd., New York

Virologic and Immunologic Characterization of Symptomatic and Asymptomatic Primary HIV-1 Infection

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Summary: To define virologic and immunologic differences in patients with acute symptomatic and asymptomatic primary human immunodeficiency virus type 1 (HIV-1) infection, sequential plasma specimens were obtained longitudinally for 1–2 years postseroconversion from subjects with well-documented time of seroconversion. Thirteen of them had an acute symptomatic primary infection, eight subjects had asymptomatic primary infection and long-term follow-up, and 27 had asymptomatic seroconversion and short-term follow-up. Quantitative plasma HIV-1 RNA levels, $CD4^+$ lymphocyte counts, and levels of antibodies to gp120, p66, p41, p31, p24, and p17 were measured. At the time of seroconversion, there was no significant difference in HIV-1 RNA levels and $CD4^+$ counts between symptomatic (n = 13) and asymptomatic (n = 27) subjects. Subsequently, however, establishment of low levels of plasma HIV-1 RNA was seen significantly more frequently in asymptomatic (n = 8) than in symptomatic (n = 13) primary infection; this correlated with higher levels of some (anti-gp120 and anti-p31) anti–HIV-1 antibodies and a slower decline in $CD4^+$ lymphocyte counts. These results indicate that immunologic control of viremia early after infection may be a critical determinant to subsequent clinical course of HIV-1 infection. They also suggest that persons with acute symptomatic primary infection may generally progress to having acquired immune deficiency syndrome (AIDS) more rapidly than people with low-grade symptoms or asymptomatic primary infection. **Key Words:** Primary HIV-1 infection—Seroconversion—Symptomatic—Asymptomatic.

Primary human immunodeficiency virus type 1 (HIV-1) infection describes the clinical period following virus infection and preceding antibody seroconversion, during which intense viral replication and widespread dissemination occur. During this period, which generally lasts 2–6 weeks, individuals may present with clinical symptoms including fever, lymphadenopathy, pharyngitis and rash, as well as other clinical findings (1–6). The proportion of infected individuals having symptomatic primary infection is unclear. Some studies have reported a history of symptoms during primary infection in 50–70% of infected people (1), whereas others reported symptoms in <20% of cases (2,3).

Symptomatic primary HIV-1 infection has been associated with relatively rapid progression to HIV disease (2,4). Detailed virologic and immunologic studies have been performed at the time of symp-

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Manuscript received August 5, 1994; accepted January 10, 1995.

tomatic seroconversion (5,6). In asymptomatic seroconverters, however, the early evolution of virologic and serologic markers has not been extensively described because of the difficulty in identifying these patients and obtaining specimens at the time of seroconversion. Consequently, the results obtained from symptomatic and asymptomatic seroconverters have not been directly compared. We present a comparative analysis of plasma viral RNA levels, $CD4^+$ lymphocyte counts, and semiquantitative measurements of specific anti–HIV-1 antibodies after primary infection in these two groups.

METHODS

Patients and Specimens

Symptomatic Subjects

Sequential blood samples were obtained from 13 subjects who presented with severe clinical symptoms of primary HIV-1 infection. Ten subjects were homosexual men, one was a male intravenous drug user, and two were women infected by heterosexual contact. The clinical presentations are listed in Table 1. All subjects presented with at least three of the symptoms listed, most often including fever. The acute symptomatic phase of infection lasted from 1 to 5 weeks. Additional details on clinical features as well as some virologic and immunologic data at the early stages of infection have been previously reported for seven of these subjects (5,6).

Overall, 90 samples (5–16 per subject) were studied. Sixty-two (69%) of them (5–12 per subject) were collected within 90 days of primary infection, and the remaining 28 were obtained 4–22 months after seroconversion.

Asymptomatic Subjects

Sequential blood specimens were obtained from 65 homosexual men who seroconverted to HIV-1 while enrolled in two prospective studies (7,8). Although these subjects were followed-up every 6 months, we were able to accurately establish time of seroconversion for a subset of them as follows. Retrospective analyses of cellular extracts using DNA polymerase chain reaction (PCR) were performed to detect HIV-1 infection in samples obtained 6 months prior to seropositivity by conventional, viral lysate–based enzyme immunoassay (EIA) (7,8).

Eight subjects were found positive by HIV-1 DNA PCR in the last seronegative sample and were included in the present study. As expected for early seroconversion, five of the eight specimens had a restricted positive pattern of Western blot reactivity (Cambridge Biotech, Rockville, MD, U.S.A.) (p24 and gp120/160 only), one was indeterminate (gp120/160 only), and two were negative. Each sample was then further tested with a third-generation, recombinant HIV antigen–based EIA (Abbott Laboratories, North Chicago, IL, U.S.A.), which can detect both IgM and IgG anti-HIV antibodies (9). Six of eight samples were positive for HIV antibody with the third-generation EIA.

The window period of PCR positivity and third-generation EIA negativity has been estimated at ~1 week, and that of third-generation EIA positivity and conventional (first generation) viral lysate-based EIA negativity at another week (9,10). Thus, the first PCR-positive sample collected from each of the eight asymptomatic individuals, like the samples obtained from the symptomatic seroconverters, had been obtained at a time that was probably very close (i.e., within 1–2 weeks) to the time of seroconversion by viral lysate–based EIA.

At each visit, subjects underwent a physical examination and phlebotomy. None of the eight individuals reported symptoms consistent with primary HIV-1 infection, either at the time of HIV-1 DNA PCR positivity or in the 6-month period preceeding and following viral detection by PCR.

TABLE 1. *Clinical presentation of subjects with acute symptomatic primary infection*

Symptoms	Frequency
Fever	12
Sore throat	9
Rash	6
Nausea/vomiting	3
Adenopathy	3
Headache	3
Diarrhea	2
Myalgia	2
Meningitis	1
Thrush	1
Encephalitis	1

Asymptomatic Plasma Donors

Because only one sample was obtained around the time of seroconversion in asymptomatic seroconverters, the level of markers of viremia (p24 antigen and HIV-1 RNA) most likely would not represent peak level because of the rapid changes in plasma virus titers at the early stages of seroconversion (5,6). Therefore, determinations of peak p24 antigen and HIV-1 RNA levels were made on another group of 27 asymptomatic seroconverters from whom serial samples (two to three per week) were obtained prior to and during the time of seroconversion, but who lacked more than on average 30 days of subsequent clinical follow-up (9,11). The frequency of samples collected at the time of primary infection was similar to that of symptomatic seroconverters. For each subject, a rise in p24 antigen values was observed before antibodies could be detected, followed by a decline to undetectable levels. The sample having the highest concentration of p24 antigen determined the "peak" value.

Each person was subjected to a pre-donation questionnaire including detailed health status and risk factors similar to that of blood donors. In addition, body temperature was measured and subjects with >37.5°C were deferred from donating. None of 27 individuals who seroconverted to HIV-1 reported risk factors or clinical features (sore throat, cold, coughing, skin infection/lesions, or nausea) that might indicate primary HIV-1 infection and would defer their donation.

Serologic and Virologic Assays

HIV p24 antigen was detected with a commercial sandwich-based polyclonal EIA (Abbott) without immune complex disso-

ciation treatment of the samples. Quantitation was obtained by comparison to a calibrated external standard curve as recommended by the manufacturer (package insert).

Detection of virion-associated RNA in 50 μl of plasma was obtained by immunocapture with anti-gp120/41–coated microparticles, direct lysis, and reverse transcription/amplification, as previously described (12,13). Quantitation of results was obtained by comparison to $\log_{10}$ dilutions of a calibrated external plasma standard, corresponding to $\sim 10^5$, 10^4, and 10^3 copies of HIV-1 RNA per milliliter of plasma. The absolute sensitivity of the assay was ~100 copies of HIV-1 RNA or 1,000 virions/ml (50 virions/50 μl) and was determined by testing serial dilutions of cell-free HIV-1 calibrated by electron microscopy in seronegative plasma. A ½ to 1 $\log_{10}$ difference in copy number can be accurately and consistently detected by this assay (13). For samples producing signals of intensity greater than that of 10,000 copies of HIV-1 RNA, which is outside the linear range of the assay, we determined the HIV-1 RNA titer by serial dilution of patient specimens in seronegative plasma. All samples were analyzed under code.

Detection and semi-quantitation of HIV antibodies was obtained with an automated dot-blot immunoassay, with use of recombinant proteins gp120 [expressed in CHO cells], and p66, p41, p31, p24, and p17 (all expressed in *Escherichia coli*) (Abbott) (14). Results were expressed in standard units, as the inverse natural log (− ln) of the reflectance divided by the cutoff of the assay.

Flow Cytometry and Statistics

Fresh peripheral blood mononuclear cells were stained with commercial monoclonal antibodies (Becton-Dickinson, Mountain View, CA, U.S.A.) and analyzed by flow cytometry within 6 hours of blood draw. The absolute number of $CD4^+$ lymphocytes was determined by multiplying the total lymphocyte cell count by the fraction of lymphocytes bearing the specific antigen.

Statistical analysis was performed by using the Wilcoxon's nonparametric rank sum test.

RESULTS

Peak Level of Viremia and CD4 Cell Count During Seroconversion

For subjects with symptomatic primary infection and asymptomatic plasma donors, multiple specimens were obtained around the time of seroconversion. Thus, peak level of viremia could be assessed and compared with relatively good precision in these two groups. All 27 plasma donors who evolved from seronegative to seropositive had a measurable peak of p24 antigen. The mean peak value of p24 antigen was 123 pg/ml (range, 5 to >200 pg/ml); for seven (25%) subjects the peak value was >200 pg/ml. Of the 13 symptomatic seroconverters, 11 had measurable p24 antigen prior to and during seroconversion; the remaining two subjects were positive for antibody and negative for p24 antigen at their first visit. The mean p24 antigen peak value for the nine subjects with detectable p24 antigen was 90 pg/ml (range, 20 to >200 pg/ml); three (27%) subjects had peak values >200 pg/ml.

There is a close parallel between the rise and fall in p24 antigen and HIV-1 RNA levels at the time of seroconversion (5,6,11,15). Thus, we measured peak values of HIV-1 RNA only in a subset of samples that had peak levels of p24 antigen. The mean peak HIV-1 RNA level was similar in both groups: $10^{5.5}$ in plasma donors and $10^{6.0}$ in symptomatic antigenemic seroconverters ($p = 0.215$), with values ranging from 10^4 to 10^7 copies/ml in each group.

At the time of seroconversion, the mean $CD4^+$ lymphocyte count for the 13 symptomatic subjects was 653 cells/μl (range, 140–1,200 cells/μl). $CD4^+$ lymphocyte counts for the seroconversion sample were available for only three of eight asymptomatic homosexual men (650, 1,309, and 725 cells/μl, respectively).

Evolution of Markers Following Primary Infection

The evolution of $CD4^+$ lymphocyte counts and plasma HIV-1 RNA levels over time for symptomatic ($n = 13$) and asymptomatic ($n = 8$) seroconverters with long-term follow-up is summarized in Table 2. The profiles of two representative subjects from each group are presented in Fig. 1. Within 2 months of seroconversion, all subjects who were tested for p24 antigen throughout that time became negative. Within 3–6 months of seroconversion, mean HIV-1 plasma RNA titers declined to levels that were one-tenth as high in asymptomatic as in symptomatic seroconverters ($10^{3.2}$ vs. $10^{4.2}$, respectively, $p = 0.047$) (Table 2). One of the 13 symptomatic subject appeared different from the group, since he maintained a $CD4^+$ lymphocyte count twofold higher than the average of his group (1,100 vs. 653 cells/μl, respectively) and his plasma HIV-1 RNA decreased to undetectable levels. Similarly, after 6 months, one of the eight asymptomatic subjects had rapidly declining levels of $CD4^+$ lymphocytes (170 cells/μl) clearly different from the other subjects of this group (mean, 1,020 cells/μl; range, 740–1,900 cells/μl). This person also had persistent high levels (10^4 copies/ml) of plasma HIV-1 RNA. These two subjects were designated as "atypical."

At 12 and 18 months postseroconversion, the difference in HIV-1 RNA levels between the two groups was notable but only of borderline statistical

TABLE 2. *Evolution of mean CD4+ lymphocyte count and plasma human immunodeficiency virus type 1 (HIV-1) RNA titer over time in symptomatic and asymptomatic seroconverters*

Time	Symptomatic	Asymptomatic	p Value
CD4+ lymphocyte count (mean number/μl)			
Seroconversion	653 (n = 13)	894 (n = 3)	N/A
<3 months	665 (n = 10)	ND	N/A
6–12 months	420 (n = 6)	730 (n = 8)	.141
	309[a]	824[a]	.005[a]
14–18 months	490 (n = 7)	680 (n = 8)	.160
	415[a]	774[a]	.022[a]
Plasma HIV-1 RNA (mean copies/ml)			
Seroconversion	$10^{6.0}$ (n = 11)	$10^{5.5}$[b] (n = 13)	.21
<3 months	$10^{4.1}$ (n = 12)	ND	N/A
6–12 months	$10^{4.2}$ (n = 7)	$10^{3.2}$ (n = 8)	.047
	$10^{4.5}$[a]	$10^{3.1}$[a]	.017[a]
14–18 months	$10^{3.8}$ (n = 7)	$10^{3.0}$ (n = 8)	.060
	$10^{4.1}$[a]	$10^{2.8}$[a]	.037[a]

All symptomatic subjects contributed to the measurements but results at specific times were obtained with the number of subjects shown in parentheses.

ND, not done; N/A, not applicable.

[a] Indicates results obtained after removing the two atypical subjects described in the text.

[b] Indicates results obtained from asymptomatic plasma donors (see text).

significance (p = 0.047 and 0.060, respectively). However, statistical significance was more pronounced (p = 0.017 and 0.037, respectively) if the two atypical subjects were removed from the analysis (Table 2). Five of eight (62%) asymptomatic seroconverters had persistent low levels (10^3 or fewer copies/ml) of plasma RNA for at least 2 years after infection. Conversely, of the seven symptomatic seroconverters from whom samples were obtained 1–2 years after infection, five (71%) had persistent RNA levels of at least 10^4 copies/ml.

Between 6 and 18 months after seroconversion, the mean CD4+ lymphocyte count was lower in symptomatic than in asymptomatic seroconverters. Because of the small number of subjects, the difference at 12 or 14–18 months was not significant between the two groups (p = 0.141 and 0.168, respectively). However, it reached statistical significance (p = 0.005 and 0.022 at 6–12 and 14–18 months, respectively) when the two atypical subjects were removed from the analysis. From 6 to 18 months of follow-up, mean CD4+ lymphocyte count was lower than 500/μl in seven of nine (78%) symptomatic but only two of eight (25%) asymptomatic seroconverters.

Evolution of Antibody Levels over Time

At ~1 year postseroconversion, the levels of antibody to gp120 and p31 tended to be weaker in symptomatic than in asymptomatic seroconverters (Table 3). However, statistical significance was not reached, probably because of the small number of subjects in each group. Three of eleven (27%) symptomatic, versus seven of eight (87%) asymptomatic, seroconverters had levels of anti-gp120 antibody >9 standard units (p = 0.079, Wilcoxon's rank sum test). Similarly, seven of eleven (63.6%) symptomatic, versus eight of eight (100%) asymptomatic subjects had anti-p31 values >12 standard units (p = 0.059). The mean level of antibody to p66, p41, p24, and p17 was essentially identical in both groups (Table 3).

DISCUSSION

The frequency and clinical significance of p24 antigenemia during the early stages of seroconversion is not well defined (1,2,4,9). Our results indicate that p24 antigen is present transiently at the early stages of most, if not all, primary infections regardless of clinical presentation. This suggests that detectable antigenemia at the time of seroconversion is a general feature of HIV-1 infection. In addition, the mean peak levels and ranges of p24 antigen values were similar in symptomatic and asymptomatic seroconverters, as were the peak levels of HIV-1 RNA. A similar peak of primary viremia was also observed in a recent longitudinal study of rhesus macaques who had different rates of disease progression (15). In contrast, the persistent levels of viremia established after primary infection correlated with rapid progression (15). Together, these results indicate that the quantitative level of viremia during the dissemination phase is unlikely to be a primary determinant of symptoms or to be predictive of disease progression.

Several studies have reported that symptomatic seroconverters tend to progress to AIDS more rapidly than do asymptomatic seroconverters (1,2,4). In addition, recent data have revealed that, within infected individuals, the level of circulating infected peripheral blood mononuclear cells and of plasma HIV-1 RNA are established within several months of seroconversion and remain relatively constant throughout subsequent HIV-1 infection (16,17). Moreover, the level of persistent viremia correlates with clinical progression (16,17). The results of the present study are in agreement with these findings. Although we analyzed a small number of individuals, we found that symptomatic primary infection was generally associated with a persistently higher

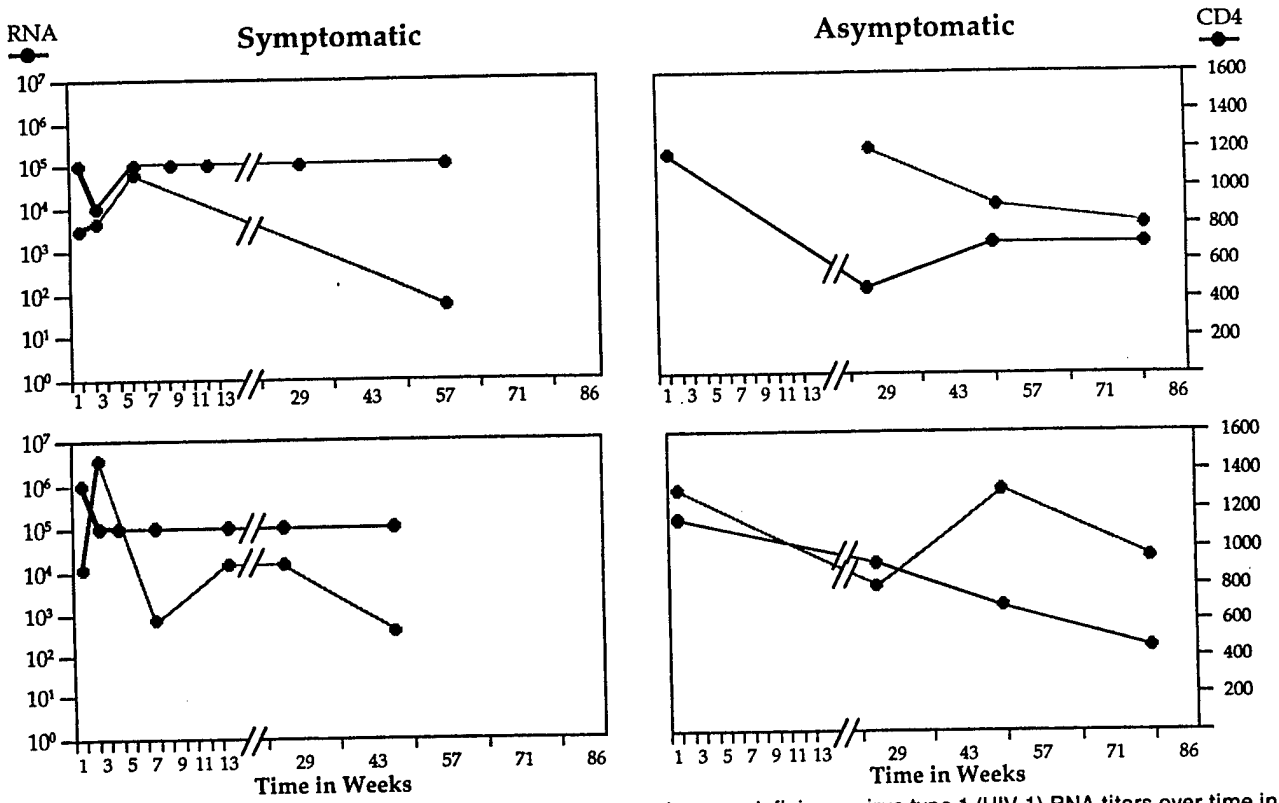

FIG. 1. Evolution of CD4$^+$ lymphocyte count and plasma human immunodeficiency virus type 1 (HIV-1) RNA titers over time in two representative symptomatic (left panel) and asymptomatic (right panel) seroconverters. HIV-1 RNA levels are indicated in black and CD4 counts in gray.

level of plasma viremia, a faster rate of CD4$^+$ lymphocyte count decline, and a weaker humoral response to some HIV-1 proteins than was asymptomatic primary infection. Thus, acutely symptomatic primary HIV-1 infection may be associated with a faster clinical progression due, at least in part, to less effective immunologic control of the initial infection. These findings also raise caution regarding interpretation of studies based solely on symptomatic seroconverters, which may not reflect general features of HIV-1 infection.

Although our observations indicate that symptomatic seroconversion may be generally associated with a more rapid clinical course of HIV infection, it is important to recognize that there is not an absolute correlation. For example, one asymptomatic seroconverter completely failed to reduce RNA titer after seroconversion and had very rapidly declining CD4$^+$ lymphocyte counts. Conversely, one symptomatic seroconverter established very low, persistent levels of plasma HIV-1 RNA and experienced a slow decline in CD4$^+$ cell count. Because the results of laboratory markers for these subjects

TABLE 3. *Levels (standard units) of specific human immunodeficiency virus type 1 (HIV-1) antibody at ~1 year postinfection for eleven symptomatic and eight asymptomatic subjects*

Subject	Time (months)	Antibody to p120	p66	p41	p31	p24	p17
Symptomatic							
1	12	13	8	22	15	8	2
2	7	10	10	23	14	11	0
3	12	10	7	21	14	12	4
4	14	8	6	18	13	2	1
5	10	7	2	20	9	10	0
6	14	6	8	20	9	11	5
7	11	6	2	20	9	2	0
8	12	6	6	22	13	3	1
9	12	5	7	21	12	12	4
10	18	1	10	21	15	10	0
11	10	0	0	17	0	4	0
Asymptomatic							
1	12	12	3	21	12	11	0
2	12	11	10	21	16	11	2
3	12	11	2	20	15	12	4
4	12	10	9	20	15	3	1
5	12	10	11	21	15	11	4
6	12	10	3	21	13	11	0
7	12	9	6	20	16	10	5
8	12	0	0	19	12	2	0

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 11, Number 3, 1995
Mary Ann Liebert, Inc., Publishers

Clinical Evaluation of Branched DNA Signal Amplification for Quantifying HIV Type 1 in Human Plasma

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ABSTRACT

Quantification of HIV-1 RNA in human plasma has provided unique insight into AIDS pathogenesis and promises to hasten progress in antiretroviral therapy and vaccine research. However, no generally available HIV-1 RNA assay has yet been subjected to rigorous clinical testing or to comparative evaluation with research-based RNA assays using large numbers of well-characterized clinical specimens. In this study, the Chiron Quantiplex branched DNA (bDNA) signal amplification assay was used to measure viral RNA in the plasma of 152 HIV-1-positive individuals at all stages of infection and in 12 patients before and after initiating zidovudine therapy. Eighty-six percent of patients had bDNA assay results above the 10,000-RNA Eq/ml sensitivity cutoff. Branched DNA values were significantly correlated with plasma viral RNA levels determined by quantitative competitive polymerase chain reaction (QC-PCR) assay (Spearman rank correlation, $r = 0.89$), infectious plasma virus titers ($r = 0.72$), p24 antigen levels ($r = 0.51$), immune complex dissociated p24 antigen levels ($r = 0.56$), and CD4$^+$ lymphocyte counts ($r = -0.72$; $p < 0.0001$ for all comparisons). Plasma viral RNA determinations by bDNA and QC-PCR assays were quantitatively similar in the range of 10^4 to 10^7 RNA molecules/ml [log bDNA = 0.93 + 0.80 (log QC-PCR); $R^2 = 0.81$, $p < 0.0001$] and declined identically following the institution of zidovudine therapy (68–73% decrease from baseline). The close quantitative correlation between bDNA and QC-PCR results, and their significant association with other viral markers and CD4$^+$ counts, support the use of plasma viral RNA measurement in HIV-1 clinical trials.

INTRODUCTION

PERSISTENT VIRAL REPLICATION is now recognized for its central role in human immunodeficiency virus type 1 (HIV-1) pathogenesis and natural history.[1–8] Viral load determinations in plasma, peripheral blood mononuclear cells (PBMCs), and lymphoid tissue have been significantly correlated with clinical stage and CD4$^+$ lymphocyte counts, with the highest viral levels occurring in primary (acute) and late-stage infection and lower levels occurring in early and intermediate stages.[1–13] Certain viral measurements such as p24 antigen (p24 Ag) levels have been shown to have prognostic value,[14,15] but their utility as surrogate markers for clinical end points in therapy trials remains to be proved.[16,17]

A number of different viral markers have been evaluated as potentially valuable clinical indicators of disease activity as well as for possible surrogate markers of clinical end points (reviewed in Ref. 16). These include quantitative viral cultures of PBMCs and plasma,[1–3] p24 antigen (Ag) and immune complex dissociated (ICD) p24 Ag,[13–15,17–21] and polymerase chain reaction (PCR) amplification of PBMC-associated viral DNA and RNA.[22–26] All of these markers are directly linked to the underlying HIV disease process and thus could be expected to provide clinically relevant information. Yet, each of these assays has significant theoretical or practical limitations. For example, PBMCs comprise only a small proportion (~2%) of total lymphoid tissue[27] and may contain transcriptionally latent or defective provirus.[23–26,28–32] When stimulated in culture,

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cells expressing viral proteins are targeted for destruction or suppression by autologous HIV-specific T lymphocytes.[33,34] Quantitative virus cultures for measuring plasma viral load are similarly compromised by a high and variable proportion of defective virus[8] and by the effects of neutralizing antibodies.[35] Furthermore, quantitative cultures are costly, time consuming, and associated with substantial exposure to infectious virus. Another viral marker, p24 Ag, is released from cells either as a virion component or as non-virion-associated antigen.[36,37] p24 measurements thus reflect a combination of virus production, provirus expression, and even p24 Ag release from dying cells. Moreover, p24 Ag is often complexed with circulating anti-p24 antibody and is only variably detected even by the ICD-p24 Ag assay.[16,18,19]

There has been growing recognition of plasma virion-associated RNA as a direct, sensitive, and quantifiable measure of viral load.[8,9,23,38,39] In theory, plasma viral RNA should reflect virus production throughout the entire lymphoreticular system rather than the comparatively minor PBMC compartment. Viral RNA determinations are not affected by the biologically complex requirements of virus culture and propagation *in vitro* or by the variabilities associated with p24 Ag production and measurement. A number of different PCR, T7 RNA polymerase, and branched DNA (bDNA) signal amplification assays for quantifying viral RNA in plasma have been developed.[8,9,23,38–51] Preliminary evaluation of these assay methods has generally yielded comparable data indicating that plasma viral RNA can be detected in most infected subjects, that levels are related to clinical stage and CD4 counts, and that institution of antiretroviral therapy results in declines in viral RNA.

The Quantitative Virology Working Group of the NIH/-NIAID AIDS Clinical Trials Group (ACTG) conducted a blinded, multicenter evaluation of six different quantitative plasma viral RNA assays (Chiron [Emeryville, CA] bDNA assay,[49] Roche [Nutley, NJ] RT-PCR assay,[44] Abbott [North Chicago, IL] immunocapture PCR assay,[50] and three noncommercial RT-PCR assays [Ref. 9, and unpublished data]) and concluded that several of them exhibited the requisite sensitivity, specificity, and precision to proceed with their evaluation in clinical trials.[39] Two of these assays, the Chiron bDNA and Roche RT-PCR assays, are commercially available as an onsite test system at clinical research centers and are beginning to be used in clinical trials of novel therapeutics.[4] Despite the introduction of the bDNA assay and other commercially available assays for HIV-1 plasma RNA quantitation into clinical trials, scant correlative information has been published describing the relation of HIV-1 RNA levels determined by these commercial assays with respect to clinical stage, $CD4^+$ counts, other conventional virological markers, or response to antiretroviral therapy. Moreover, no studies have been published directly comparing two independent HIV-1 RNA assays using large numbers of well-characterized clinical samples as a means to cross-validate the respective methodologies and to better estimate the actual levels of circulating virus. The present study was thus designed to address three objectives: (1) to evaluate the performance characteristics of the bDNA assay in patients representing the complete clinical spectrum of HIV-1 infection, (2) to define the quantitative relationship between viral load measurements determined by bDNA assay and those determined by quantitative competitive-polymerase chain reaction (QC-PCR) assay,[8] quantitative plasma culture, and regular and ICD-p24 Ag testing, and (3) to determine the magnitude and kinetics of change in bDNA-determined viral RNA levels compared with other viral markers in patients treated with zidovudine.

MATERIALS AND METHODS

Patients

Peripheral blood specimens were obtained with consent from a total of 271 study subjects. At the Aaron Diamond AIDS Research Center (ADARC, New York, NY), blood was collected from 80 randomly selected HIV-1-seropositive individuals seen at New York University Medical Center, the New York Blood Center, and Bellevue Hospital. The subjects included patients with acute HIV-1 infection (2 cases), AIDS (3 cases), AIDS-related complex (ARC; 10 cases), and asymptomatic infection (65 cases). $CD4^+$ lymphocyte counts ranged from 4 to 1001 cells/mm^3. Antiretroviral therapy experience in these individuals was unknown. Plasma specimens obtained from 90 HIV-1-seronegative subjects served as controls. Plasma samples were prepared from blood specimens collected in heparin that were centrifuged at $400 \times g$ for 10 min at room temperature and stored at −80°C until analysis. At the University of Alabama at Birmingham (UAB, Birmingham, AL) plasma specimens for bDNA analysis were obtained from 72 HIV-1-seropositive subjects, using archived samples remaining from 2 previous clinical studies.[8,52] In the first instance, replicate frozen (−70°C) plasma samples from each of 66 subjects previously evaluated for HIV-1 plasma viral RNA by the QC-PCR method in a study of HIV-1 natural history[8] were analyzed. In the second instance, sequential plasma samples from 12 subjects who represented the zidovudine (azidothymidine, AZT) control arm of a prospective, randomized, double-blinded phase I/IIA study of the nonnucleoside analog reverse transcriptase inhibitor L-697,661[52] were examined. Baseline samples from 6 of these longitudinally evaluated patients were included in the natural history study (see above), bringing the total UAB study population to 72. Specimens used for bDNA analysis were unselected and represented the entire sample sets for patients enrolled consecutively into the respective studies. The UAB subjects included 6 patients with acute infection, 25 with asymptomatic infection, 24 with ARC, and 17 with AIDS. $CD4^+$ lymphocyte counts ranged from 5 to 1080 cells/mm^3. Thirteen subjects were receiving antiretroviral therapy consisting of either zidovudine or didanosine at the time of study. Twenty-nine control samples were obtained from healthy, uninfected volunteers. Plasma specimens at UAB were derived from blood samples that had been collected in acid citrate dextrose (ACD) and processed by sequential 15-min centrifugations at $200 \times g$ and $1000 \times g$ prior to storage at −70°C in order to ensure the removal of the majority of platelets.

p24 antigen assay, quantitative culture, and QC-PCR

Plasma p24 antigen levels were determined by commercial enzyme immunoassays (ADARC [Abbott Laboratories]; UAB [Coulter Diagnostics, Hialeah, FL]). Sensitivity cutoff values for the p24 antigen assays were 5 pg/ml for the regular assay

and 15 pg/ml for the ICD method. The quantitation of infectious HIV-1 titers in plasma was performed using the end point-dilution culture method as described previously.[1,3] Levels of HIV-1 RNA in plasma for all UAB specimens were determined at Genelabs Technologies (Redwood City, CA) by the QC-PCR method.[8] The QC-PCR, infectious plasma virus titers, and p24 antigen results for the UAB subjects have been reported previously.[8,53]

Branched DNA assay

HIV-1 RNA in plasma was quantified at the ADARC and UAB study sites, using the branched DNA signal amplification method (Quantiplex HIV RNA assay; Chiron) as described.[45,49] All plasma specimens were coded and blinded to individuals performing the assay. Virus was concentrated from duplicate plasma specimens as recommended by the manufacturer by centrifugation in a bench top microcentrifuge (centrifuge model 17RS, rotor 3753; Heraeus) at 23,500 × *g* for 1 hr. Because of limited sample volumes, 0.25 to 1.0-ml specimens were initially used, adjusting their total volume as necessary to 1.0 ml with normal human plasma, and correcting the final bDNA readout by the corresponding dilution factor. This approach was shown experimentally not to affect the quantitative results of the bDNA assay except for its threshold sensitivity (our unpublished data). For specimens yielding a negative bDNA result and for which less than the recommended duplicate 1.0-ml plasma volumes were initially tested, the assay was repeated using 1.0-ml samples. The concentration of RNA in a specimen, expressed as HIV RNA equivalents (Eq) per milliliter, was determined from a standard curve with a dynamic range of 10^4 to 1.6×10^6 RNA Eq/ml of plasma, as described.[45] Samples containing less than 10^4 RNA Eq/ml were recorded as nondetectable and those exceeding 1.6×10^6 were diluted 1:10 or 1:100 in negative plasma and reassayed.

Statistical analysis

Descriptive statistics as well as nonparametric analyses were used to evaluate the data.[54,55] Spearman rank correlations and Pearson linear correlations were performed. Linear and nonlinear regression analyses were employed to define quantitative relationships between variables. The chi-square test was used to compare detection frequencies of various virological assay measurements in relation to patient groups exhibiting $CD4^+$ cell ranges of <200, 200–500, and >500/mm^3. The Kruskal–Wallis test was used to compare viral marker levels among the different groups, and the Wilcoxon signed rank test was used for one-sample or paired two-sample data analysis to assess the significance of treatment-related viral load changes.

RESULTS

Branched DNA signal amplification of plasma viral RNA, quantitative plasma virus cultures, and regular p24 Ag assays were performed on plasma specimens from a total of 152 HIV-1-seropositive patients at the two study sites. Represented in this group of patients were 8 subjects with acute (primary) infection and 144 subjects with chronic infection. Eighty-six percent (131 of 152) of all patients had detectable bDNA values above the 10,000-RNA Eq/ml assay sensitivity cutoff. Branched DNA values for plasma viral RNA ranged from $<10^4$ Eq/ml to 9×10^7 Eq/ml. Fifty-eight percent (88 of 152) of patients had culturable plasma virus and 46% (70 of 152) had measurable p24 Ag. QC-PCR analysis of plasma viral RNA and ICD-p24 Ag testing were performed on specimens from the 72 patients at the UAB site (6 with acute infection and 66 with chronic infection). The sensitivities of QC-PCR and ICD-p24 Ag assays were 100% (72 of 72) and 61% (44 of 72), respectively. QC-PCR-determined RNA values ranged from 10^2 to 2×10^7 mol-

TABLE 1. COMPARISON OF VIROLOGICAL ASSAY DETECTION RATES IN DIFFERENT STAGES OF HIV-1 INFECTION[a]

Site	CD4 count	Number tested	Plasma culture	p24 Ag		Plasma viral RNA	
				Regular	ICD	QC-PCR	bDNA
ADARC	<200	52	45 (87)[b]	26 (50)	ND	ND	47 (90)
	200–500	18	6 (33)	3 (17)	ND	ND	14 (78)
	>500	8	0 (0)	0 (0)	ND	ND	6 (75)
	Subtotal:	**78**	**51 (65)**	**29 (37)**	ND	ND	**67 (86)**
UAB	<200	31	24 (77)	26 (84)	27 (87)	31 (100)	30 (97)
	200–500	19	5 (26)	6 (32)	8 (42)	19 (100)	15 (79)
	>500	16	0 (0)	1 (6)	3 (19)	16 (100)	11 (69)
	Subtotal:	**66**	**29 (44)**	**33 (50)**	**38 (58)**	**66 (100)**	**56 (85)**
All sites	Total:	**144**	**80 (56)**	**62 (43)**	**38 (58)**	**66 (100)**	**123 (85)**

[a]Patients with primary (acute) HIV-1 infection were excluded from this analysis.
[b]Number and (percentage) of subjects with positive assay results.
Abbreviation: ND, Not done.

ecules/ml. None of 119 HIV-seronegative control subjects had positive marker results by any of the assays used.

Table 1 shows the relative sensitivities of the bDNA assay compared with the other assay methods as a function of $CD4^+$ lymphocyte counts in patients with chronic infection. For this analysis, the eight subjects with acute illness were excluded because such patients are uniformly viremic and have viral load measurements that bear no consistent relationship to CD4 counts.[8,12,13] Eighty-five percent of patients had viral RNA detectable by bDNA compared with 100% by QC-PCR and 56% by viral culture. Forty-three percent of all subjects, and 50% of UAB subjects, had detectable p24 Ag. The proportion of the latter patient group which had p24 Ag detectable by the ICD method was 58%. Except for QC-PCR, which was positive in all subjects tested, there was an obvious, highly significant inverse correlation between CD4 counts and the frequency of detection by each virological marker ($p < 0.001$ for all). The mean (±1 SD) of bDNA values for plasma viral RNA in patients with $CD4^+$ cell counts of $<200/mm^3$, 200–500/mm^3, and $>500/mm^3$ were 533 (±201) × 10^3, 71 (±12) × 10^3, and 45 (±8) × 10^3 RNA Eq/ml, respectively. Branched DNA values for patients with $CD4^+$ cells $<200/mm^3$ were significantly greater than for patients with higher CD4 counts ($p < 0.0001$). Of the 123 bDNA-positive patients with chronic infection (Table 1), 1 had a value between 10^7 and 10^8 RNA Eq/ml, 5 had values between 10^6 and 10^7, 56 had values between 10^5 and 10^6, and 61 had values between 10^4 and 10^5 (including 13 patients with bDNA values less than 25,000 RNA Eq/ml). There were no statistically significant differences in detection rates for any of the viral markers between the two study sites except for plasma virus cultures, which were more commonly positive at ADARC (65%) than at UAB (44%) ($p < 0.05$).

The availability of replicate plasma samples from the UAB study site for which bDNA, QC-PCR, p24 Ag, ICD-p24 Ag, and quantitative viral culture results were all determined enabled us to analyze directly the quantitative relationship between bDNA values and these other viral markers. Figure 1 depicts these data, showing positive correlations between bDNA and QC-PCR, bDNA and ICD-p24 Ag, and bDNA and culture results. Table 2 summarizes the Spearman rank correlation coefficients among these viral load markers and between each of them and CD4 levels. Branched DNA and QC-PCR correlated most strongly with $CD4^+$ cells ($r = -0.72$ and -0.75, $p < 0.0001$ for both). Among the viral load markers, bDNA and QC-PCR correlated with each other most strongly whether considering all subjects ($n = 72$, $r = 0.89$, $p < 0.0001$) or only those in whom both markers were detectable ($n = 56$, $r = 0.91$, $p < 0.0001$). Pearson correlation analysis, which evaluates linear relationships, revealed a similarly strong correlation between $\log_{10}$ bDNA and $\log_{10}$ QC-PCR ($r = 0.89$, $p < 0.0001$). To evaluate further the quantitative relationship between plasma viral RNA levels determined by bDNA and those determined by QC-PCR, a linear regression analysis of the data shown in Fig. 1A was performed. The best fit line is described by the equation log bDNA = 0.93 + 0.80 (log QC-PCR) ($R^2 = 0.81$, $p < 0.0001$).

Interassay reproducibility of the bDNA assay was determined by quantifying one positive specimen and one negative specimen, in duplicate, over the course of 17 assay runs at ADARC and UAB. The overall (interlaboratory and interassay) mean and standard deviation for quantitation of the positive specimen were 66.6 × 10^3 and 13.7 × 10^3 RNA Eq/ml, respectively, resulting in a 21% coefficient of variation. In all of the 17 assay runs, the negative control quantitated below the assay cutoff of 10^4 RNA Eq/ml. In other studies (G. Shaw, unpublished), we determined the natural biological variation in plasma HIV-1 RNA levels in five untreated subjects over a period of 5 weeks. In these individuals, blood samples were obtained on days 1, 4, 8, 11, 15, 18, 22, 29, and 35, and bDNA assays were performed in batch in one laboratory. Mean bDNA levels in each of the subjects over the 5-week study period ranged from 3.8 × 10^4 to 1.1 × 10^6 RNA Eq/ml. Coefficients of variation for each subject's nine serial determinations ranged from 9.9 to 22.2% (16.6 ± 5.0%, mean ± 1 SD).

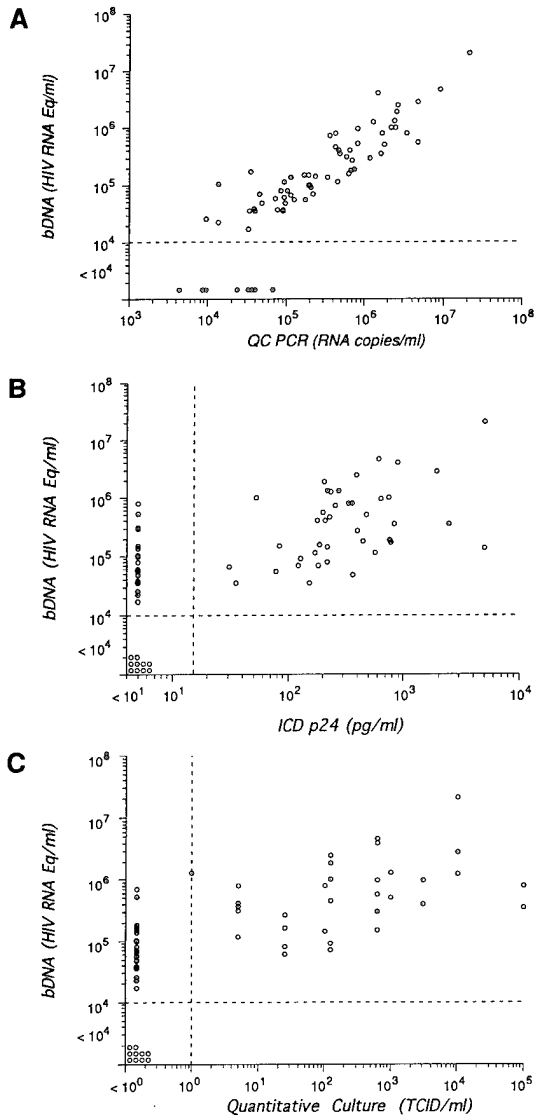

FIG. 1. Plasma viral RNA levels determined by bDNA assay compared with plasma viral RNA assayed by QC-PCR (A), plasma viral p24 Ag determined by the immune complex dissociation (ICD) assay (B), and infectious plasma virus determined by end-point culture (C). Sensitivity cutoff levels for the respective assays are represented by dashed lines.

TABLE 2. SPEARMAN RANK CORRELATIONS AMONG VIRAL LOAD MEASUREMENTS AND CD4+ LYMPHOCYTE COUNTS[a,b]

	CD4+ cells	*p24 Ag*	*ICD p24 Ag*	*Plasma culture*	*QC-PCR*	*bDNA*
CD4+ cells	—	−0.58	−0.61	−0.67	−0.75	−0.72
p24 Ag		—	0.90	0.62	0.52	0.51
ICD-p24 Ag			—	0.58	0.54	0.56
Plasma culture				—	0.76	0.72
QC-PCR					—	0.89
bDNA						—

[a]Comparisons performed on viral load data depicted in Fig. 1 along with corresponding p24 Ag data and CD4+ lymphocyte counts. Patients with primary (acute) infection and those with negative bDNA values were excluded.

[b]All correlations shown are significant at the $p < 0.0001$ level.

Having characterized the bDNA assay sensitivity (85%), specificity (100%), reproducibility (coefficient of variation, 21%), quantitative range (10^4–10^8 RNA Eq/ml), and correlation with CD4+ cell counts ($r = -0.72$, $p < 0.0001$) and four direct virological markers ($r = 0.51$ to 0.89, $p < 0.0001$ for all), we next sought to evaluate the dynamic response of the assay in the clinical setting of primary (acute) HIV-1 infection and following the institution of antiretroviral therapy. Acute HIV-1 infection is characterized by high levels of viral replication with infectious virus titers, virion-associated RNA (determined by PCR methods), and plasma p24 Ag all reported to be high prior to antibody seroconversion.[8,12,13] We thus determined plasma viral RNA levels by the bDNA method, along with infectious virus titers and p24 Ag levels, through the period of acute symptomatic infection and seroconversion in two ADARC patients (Fig. 2). Peak levels of plasma virion-associated RNA corresponding to the first available blood specimens ranged from 4×10^6 to 9×10^7 Eq/ml and fell by 100- to 10,000-fold in association with antibody seroconversion. Whether even higher viral RNA levels were present at earlier time points during acute infection cannot be determined. Infectious virus titers and p24 Ag levels paralleled those of bDNA. Plasma virus assayed by the bDNA method, but not by culture or p24 Ag assay, remained detectable throughout the 1–2 years of clinical follow-up. Branched DNA assays were also performed on plasma specimens from six patients at UAB who had acute HIV-1 infection. These determinations were made on single plasma specimens corresponding to time points of peak viremia based on QC-PCR and p24 Ag measurements.[8,56] Branched DNA values for these patients ranged from 7×10^5 to 2×10^7 RNA Eq/ml while QC-PCR measurements of replicates of the same plasma specimens ranged from 4×10^5 to 2×10^7 RNA molecules/ml. RNA results determined by the two assay methods for these six patients were highly correlated ($r = 0.87$, $p < 0.03$).

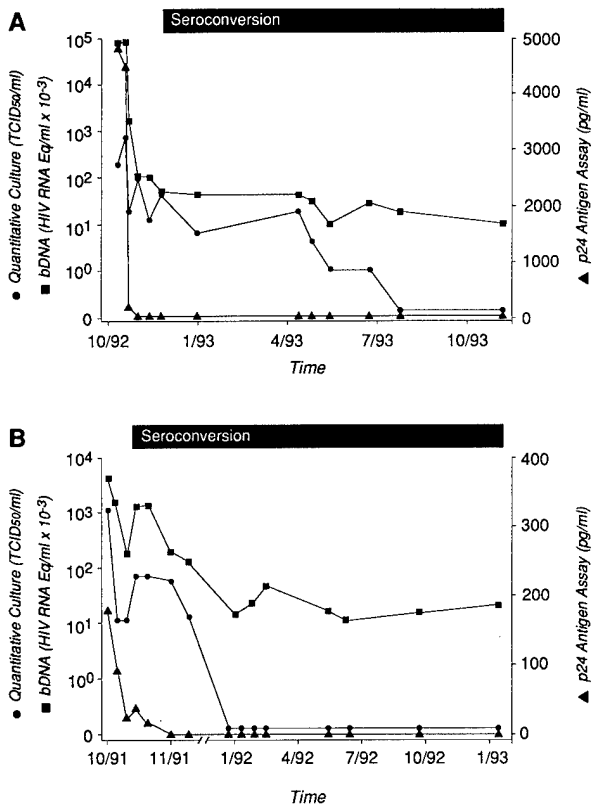

FIG. 2. Plasma viral load measurements by bDNA, p24 Ag, and end-point culture assays in two patients (A and B) with primary (acute) HIV-1 infection. The timing of first antibody detection and seroconversion is indicated by the bar (labelled *Seroconversion*). Note that bDNA values as plotted have been multiplied by 10^{-3}, so that a value plotted at 10^3 actually represents 10^6 HIV-1 RNA Eq/ml.

Finally, sequential plasma specimens from 12 patients beginning zidovudine therapy were analyzed by bDNA, QC-PCR, p24 Ag, and ICD-p24 Ag assays before, during, and 1 week after a 6-week course of drug therapy (Fig. 3). Of note, all four assays were performed on batched samples so as to minimize interassay variability. At baseline, bDNA- and QC-PCR-determined viral RNA levels were quantifiable in all 12 subjects, ranging from 4.8×10^4 to 7.9×10^5 Eq/ml for bDNA and 3.5×10^4 to 1.2×10^6 molecules/ml for QC-PCR. p24 Ag and ICD-p24 Ag were detectable at baseline in six and seven subjects, respectively. Figure 3A shows that initiation of zidovudine therapy resulted in an immediate and generally sustained fall in viral RNA as measured by the bDNA assay. Discontinuation of

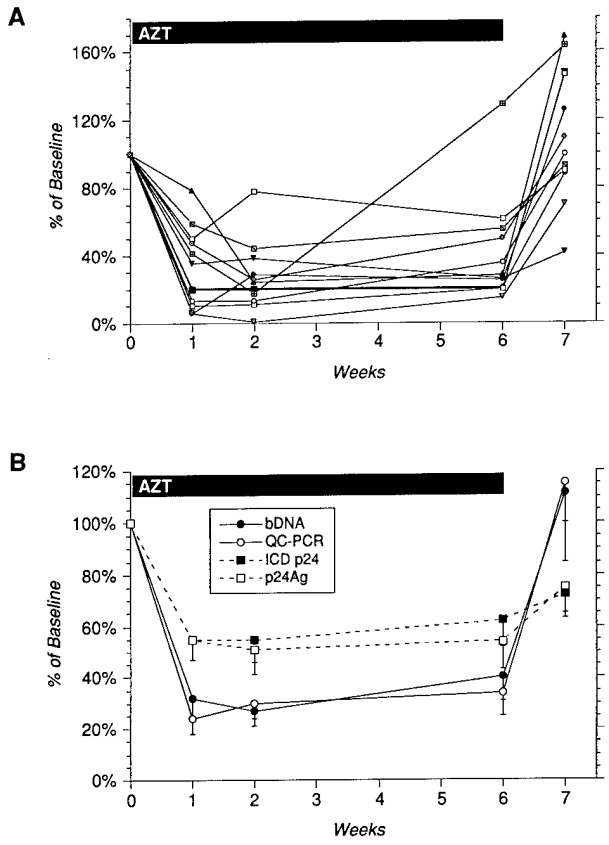

FIG. 3. Changes in plasma viral RNA determined by bDNA and QC-PCR assays and plasma viral core antigen determined by regular and ICD-p24 Ag assays following the institution of a 6-week course of zidovudine (AZT) at 500 mg qd.[52] Therapy was discontinued after week 6. Viral load measurements are plotted as a percentage of baseline (mean ± 1 SEM). Individual patient data for the bDNA assay are shown in (A) (n = 12) and combined data (mean ± 1 SEM) are shown in (B).

zidovudine after week 6 led to an immediate increase and return to baseline of viral RNA. The viral RNA responses to zidovudine therapy as measured by bDNA and QC-PCR were virtually identical in kinetics and magnitude (Fig. 3B), resulting in a statistically significant drop in both markers at week 1 to 27–32% of their baseline values ($p < 0.001$). Branched DNA levels fell to less than the assay sensitivity limits (10^4 RNA Eq/ml) in 5 subjects, whereas QC-PCR-determined levels remained quantifiable in all 12 patients (7 patients had QC-PCR nadir values below 2×10^4, including 4 patients with values below 1×10^4 RNA molecules/ml). Changes in p24 Ag and ICD-p24 Ag paralleled those for viral RNA although they were of lesser magnitude, declining at week 1 to 55% of pretreatment levels. After discontinuing zidovudine at week 6, viral RNA levels returned promptly to baseline, a change paralleled to a lesser degree by p24 Ag and ICD-p24 Ag.

DISCUSSION

A number of investigative groups have independently developed and reported novel assay methods for quantifying HIV-1 RNA in plasma.[8,9,23,38,40,44,45,47–51] In the early stages of the development of these assays, discordant results were frequently reported regarding the frequency of detection, magnitude, and stability of HIV-1 RNA in plasma.[8,9,23,24,38,40–51] More recently, primarily because of technical improvements, consistent and reproducible findings linking plasma viral RNA to HIV-1 pathogenesis and natural history have been reported.[8,9,23,38,56] Using an internally controlled quantitative competitive RT-PCR method (QC-PCR), Piatak *et al.*[8] demonstrated conclusively that (1) virion-associated HIV-1 RNA can be detected in virtually all seropositive individuals regardless of disease stage; (2) viral RNA levels generally range from 10^2 to 10^7 molecules per milliliter of plasma; (3) viral RNA levels correlate significantly with other virological load markers such as infectious plasma virus and p24 Ag, with clinical stage, and with $CD4^+$ lymphocyte counts; and (4) viral RNA levels fall significantly in association with resolution of primary (acute) HIV-1 infection and following the institution of antiretroviral therapy.

Using a different RT-PCR assay procedure, Winters *et al.*[9] reported findings similar to those of Piatak *et al.*, detecting HIV-1 RNA levels in the range of 10^2 to 10^6 molecules/ml in >95% of infected subjects. Furthermore, these investigators performed rigorous analyses of the natural biological variation of HIV-1 RNA in plasma, of assay reproducibility, and of the stability of plasma virus with long-term storage. They found a median intraassay reproducibility of $\log_{10}$ 0.15, median interassay reproducibility of $\log_{10}$ 0.25, biological variation in viral RNA levels of $\log_{10}$ 0.30 in patients on no therapy or unchanged therapy, and stability of virion-associated RNA in plasma stored at –70°C for up to 1 year. Coombs *et al.*[46] and Aoki-Sei *et al.*,[38] using still other methods for quantifying plasma virus, also demonstrated consistent and reproducible virus quantitation in fresh and stored human plasma.

On the basis of these reports and others, there has been increasing interest and concentration on the part of clinical investigators, clinicians, and patients alike to explore the use of plasma viral load measurements to assess drug activity in clinical trials and in the setting of individual patient management. In this context, however, it is important to emphasize that only the more technically demanding research-based assays described above have been evaluated clinically. While there is the expectation that commercial assays designed for use in clinical or clinical research laboratories will yield data similar to those of basic research assays, it is essential to evaluate this question formally. Lin *et al.*[39] have provided important data in this regard demonstrating that six different HIV-1 RNA assay methods, including two commercial tests (Chiron and Roche), were able to discriminate and accurately rank a constructed 10-fold dilution series of cultured HIV-1 virus spiked into normal human plasma. Furthermore, these assays could discriminate between 19 positive or negative clinical samples and between positive samples with high versus low virus loads, although method-specific differences in the quantitative results for individual patient specimens were as high as 100-fold. Nonetheless, the reproducibility of certain of the assays, including the Chiron bDNA assay, was such that an empirical fourfold difference in RNA levels could be viewed as significant and it was recommended that they be advanced to clinical trial evaluation.[39]

The present study represents one of the first large clinical research evaluations of the Chiron branched DNA signal amplification assay and it is unique in having a combination of other viral load measurements, including QC-PCR determinations of

viral RNA, available for direct comparison. In this study, we determined bDNA values in 152 patients at all stages of infection and in 119 HIV-negative controls and we related this information to clinical stage, $CD4^+$ cell counts, results of other viral load measurements, clinical course following acute infection, and response to therapy. The sensitivity of the bDNA assay in this relatively advanced patient population (83 patients with $CD4^+$ counts < 200/mm^3, 37 patients with counts from 200 to 500/mm^3, 24 patients with counts >500/mm^3 [all with chronic infection], and 8 patients with acute infection) was 86%. Its specificity was 100%, reproducibility 21% (coefficient of variation), working range for clinical samples 10^4–10^8 RNA Eq/ml, correlation (r value) with $CD4^+$ counts −0.72 ($p <$ 0.0001), and correlation with four other viral markers ranging from 0.51 to 0.89 ($p < 0.0001$ for all). Of note, the rank correlation coefficients (Table 2) were strongest between bDNA and QC-PCR (0.89), followed by bDNA and culturable virus (0.72), and then by bDNA and ICD-p24 Ag (0.56) and p24 Ag (0.51). In comparison, p24 Ag and ICD-p24 Ag were correlated at the 0.90 level. These empirical findings are consistent with expectations based on the viral components targeted by the respective assays: bDNA and QC-PCR detect total virion-associated RNA, plasma cultures detect infectious virus, and p24 Ag and ICD-p24 Ag detect the various forms of virion- and non-virion-associated core antigen.

Theoretically, results of the bDNA and QC-PCR assays should be similar or identical because they both measure viral RNA from pelleted virus. In fact, results of the two assays were highly correlated (Spearman rank and Pearson correlation coefficients of 0.89, $p < 0.0001$ for both) over a broad range of values from 10^4 to 2×10^7 viral RNA molecules/ml (Table 2 and Fig. 1). In a total of 72 patients for whom bDNA and QC-PCR data were available (Table 1 plus 6 patients with primary infection), 75% had bDNA and QC-PCR results that differed by less than 0.5 $\log_{10}$; 99% of patients had bDNA and QC-PCR results that differed by less than 1.0 $\log_{10}$. Regression analyses revealed highly significant correlations between bDNA and QC-PCR results, indicating a nearly one-to-one relationship between bDNA and QC-PCR values over a 3 $\log_{10}$ range. Further analysis revealed a small but statistically significant method-associated trend for QC-PCR results to exceed bDNA results by an average of 0.168 $\log_{10}$ ($p < 0.001$). The fact that two independent viral RNA assays, based on completely different amplification strategies and having differently prepared quantitative standards, yielded nearly the same quantitative results for clinical samples over a 3 $\log_{10}$ dynamic range is important. Such data provide independent and mutual confirmation of the quantitative values for plasma viral RNA recorded by these assays. Other reports[24,44] suggesting that plasma viral RNA levels are generally 10- to 100-fold lower than those we determined by the bDNA and QC-PCR assays likely reflect the use of different assay methods, different quantitative standards, less well-preserved clinical specimens, or substantially different patient populations. The technically demanding nature of target (PCR) and signal (bDNA) amplification assays will require the use of common assay standards by laboratories performing these assays in clinical trial settings and planning to analyze data collectively.

Overall reproducibility of the bDNA results reported in this study was estimated by quantifying replicates of a single HIV-1-positive specimen and a single negative specimen over the course of 17 assay runs at ADARC and UAB. The overall interlaboratory and interassay mean and standard deviation for the positive specimen was $66.6 \times 10^3 \pm 13.7 \times 10^3$ RNA Eq/ml, resulting in a coefficient of variation of 21%. In 3 other studies of bDNA assay reproducibility[39,45,57] in which as many as 12 different operators performed the test, overall coefficients of variation ranged from 18 to 23%. In the ACTG Virology Working Group study,[39] assay reproducibility was determined by pooled standard deviations of results on pairs of blinded patient samples. In that study, the bDNA assay exhibited the smallest pooled standard deviation, and thus the greatest reproducibility, of all tests analyzed. Taken together, the results of the five studies indicate that differences in viral RNA results of as little as twofold, within or between assay runs, would be expected to be significant at the $p < 0.05$ level.

An important question to investigators involved in many different types of clinical HIV-1 research (including antiretroviral chemotherapy, immunotherapy, natural history, pathogenesis, and vaccine efforts) is which plasma viral RNA assays are most useful for quantifying virus load *in vivo*. In our view, a qualified answer is necessary depending on the patient population under study, baseline ranges in viral load, sensitivity and accuracy of measurements required to answer the questions posed, as well as feasibility issues such as commercial availability and ease of use of an assay method. The current study provides data for the bDNA assay relating to each of these issues: Adults at all clinical stages were evaluable. Overall, 131 of 152 (86%) of subjects had detectable bDNA levels, and even in the group of patients with greater than 500 $CD4^+$ cells/mm^3, 17 of 24 (71%) had detectable bDNA values albeit at lower levels ($45 \pm 40 \times 10^3$ RNA Eq/ml, mean ± 1 SD). Assay modifications designed to enhance the sensitivity of the bDNA assay from a threshold of 10^4 RNA Eq/ml to 5×10^2 RNA Eq/ml (our unpublished data) will substantially increase the proportion of individuals with plasma viral RNA detectable by this method. Sample volume requirements (duplicate 1-ml plasma specimens) specified for the bDNA assay can be problematic for pediatric studies or certain other applications; we successfully used sample volumes as low as 0.25 ml. Accuracy of the bDNA assay over a 3 $\log_{10}$ range (10^4 to 2×10^7 RNA Eq/ml) was independently validated by direct comparison of assay results with QC-PCR-determined values using replicate plasma samples. Facility of use of the bDNA assay was confirmed by test performance at two clinical sites (ADARC and UAB).

Plasma viral RNA determinations by the Genelabs QC-PCR assay were employed for comparison with bDNA results in this study. Whereas the bDNA assay was able to detect and quantify HIV-1 RNA in the plasma of 62 of 72 UAB subjects (Table 1 plus 6 patients with acute infection), the QC-PCR assay was positive in all 72. The mean QC-PCR-determined levels of viral RNA in the 10 subjects with confirmed HIV-1 infection but undetectable bDNA values (<10^4 RNA Eq/ml) was $10^{4.10}$ RNA molecules/ml with a standard deviation of $\log_{10}$ 0.83. The accuracy of the QC-PCR assay has been determined experimentally by measuring recombinant HIV-1 RNA and DNA standards of known concentration, by quantifying viral RNA in culture supernatants for which virion particle counts were independently determined, and now by direct comparison with bDNA results on clinical samples. The interassay variability of the QC-PCR assay is 22% (coefficient of variation).[8] Because of the routine sensitivity of the QC-PCR assay of approximately

2000 RNA molecules/ml, and its ability to detect and quantify plasma viral RNA at levels as low as 100 molecules/ml on an as needed basis, this assay has proven to be particularly useful in studies in which accurate quantitation of viral RNA in the range of 100 to 50,000 molecules/ml is important.

Mulder *et al.*[44] described a Roche Molecular Systems RT-PCR assay for quantifying plasma viral RNA and its performance characteristics for detecting and quantifying HIV-1 RNA expressed from plasmid vectors and in culture supernatants. More limited published data are available regarding the performance of this assay on clinical specimens.[39,44] If the sensitivity (200 HIV-1 RNA copies/ml plasma), low sample volume requirements (200 μl of plasma), accuracy, and reproducibility reported for the research assay[44] are maintained in a commercially available assay, then it too will represent an important experimental tool for clinical AIDS research.

In summary, there are currently three commercial[8,44,45] and a number of noncommercial[9,24,38,39,47,50,51] assays available for assessing HIV-1 plasma viral load in clinical specimens. The findings reported here and elsewhere[8,9,39,44,45] suggest that at least some of these assay methods detect and accurately quantify the same viral RNA target molecule that exists in plasma in virion-associated form throughout all stages of clinical infection.[8,9,23,38,51] Studies by the ACTG Virology RNA Validation Group have, for the first time, provided evidence that therapy-induced changes in HIV-1 viral RNA load may be significantly associated with clinical outcome (ACTG protocol 116B/117; R. Coombs, personal communication). These latter findings, along with the clinical evaluation of bDNA and QC-PCR assays described here, suggest that plasma viral RNA determinations may indeed fulfill the requirements and promise of a clinically useful surrogate marker for evaluating the clinical benefits of new antiretroviral therapies.[58,59]

ACKNOWLEDGMENTS

The authors thank Sherri Campbell-Hill and Limei Yang for technical assistance, Jennifer Wilson for artwork, and Melinda Ingrum for preparation of the manuscript. This work was supported by the core research facilities of the New York University and University of Alabama at Birmingham Centers for AIDS Research and by the Birmingham Veterans Administration Medical Center. Financial support: DAMD 17-93-C-3146 (G.M.S.), NIH AI 32755 (M.S.S.), NIH AI 35467 (J.D.L.).

All subjects gave informed consent. The study was conducted in accordance with human experimentation guidelines of the U.S. Department of Health and Human Services, the Aaron Diamond AIDS Research Center, and the University of Alabama at Birmingham.

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Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology
10:139–149 © 1995 Lippincott–Raven Publishers, Philadelphia

Assessment of Antiretroviral Therapy by Plasma Viral Load Testing: Standard and ICD HIV-1 p24 Antigen and Viral RNA (QC-PCR) Assays Compared

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Summary: To assess the utility of quantitative competitive–polymerase chain reaction (QC-PCR) measurements of plasma human immunodeficiency virus type 1 (HIV-1) RNA and other viral load markers for assessment of antiretroviral therapy, we used archived cryopreserved specimens from a randomized controlled clinical trial of 135 patients ($CD4^+$ T cell count $\leq 500/mm^3$), comparing zidovudine (500 mg/day) versus the nonnucleoside reverse transcriptase inhibitor L-697,661 (50, 300, or 1,000 mg daily). We evaluated treatment-associated changes in plasma viral load by standard and immune complex–dissociated (ICD) HIV-1 p24 antigen assays, and, in a representative subset of patients (n = 46), by QC-PCR determination of virion-associated HIV-1 RNA. At baseline, HIV-1 RNA was quantifiable by QC-PCR in all patients tested (100%), whereas standard and ICD HIV-1 p24 antigen tests were positive (≥30 pg/ml) in 42% and 56%, respectively. All viral load parameters showed significant decreases from baseline within 1 week of initiation of zidovudine, as measured by standard p24 antigen assay, ICD p24 assay, and QC-PCR. At 1 week, patients treated with either 300 or 1,000 mg/day of L-697,661 showed significant decreases from baseline in plasma standard and ICD p24 antigen and QC-PCR–determined HIV-1 RNA levels. Whereas viral load decreases seen with zidovudine were sustained for the duration of treatment, plasma viral markers often returned to pretreatment levels despite ongoing L-697,661 treatment, with evidence of the emergence of drug-resistant virus. Whereas standard p24, ICD p24, and viral RNA levels changed similarly in response to treatment, the superior sensitivity and available dynamic range of plasma viral RNA assays like QC-PCR analysis provide an advantage for clinical monitoring of plasma viral load, allowing tracking of treatment-related changes even in patients with earlier stage disease and lower levels of viral load. **Key Words:** QC-PCR—HIV-1 RNA—Viral load markers—Zidovudine—L-697,661—ICD p24.

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The results of this study were presented in part at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, October 1993, New Orleans, Louisiana, U.S.A.

Manuscript received July 26, 1994; accepted January 3, 1995.

With the continuing spread of human immunodeficiency virus (HIV) infection and the lack of available definitive therapy, there is a pressing need for rapid and efficient evaluation of new experimental treatments. However, earlier diagnosis, improved supportive care, prophylactic and therapeutic interventions for specific opportunistic pathogens, and

the availability of first-generation nucleoside analogue antiretroviral treatments have made it increasingly difficult to conduct clinical trials based on clinical end points. The impact of these constraints has been magnified by the need to test a large number of potentially active agents alone and in combination. Accordingly, much attention has focused on the use of so-called surrogate markers as a means for assessing drug activity in clinical trials (1).

One of the most widely used surrogate markers of antiretroviral drug activity is the $CD4^+$ T lymphocyte count. However, changes in $CD4^+$ cell counts provide only an indirect measure of antiretroviral activity, whose correlation with viral replication and clinical outcome is incomplete (1–4). Evaluation of the amount of virus present in vivo—determination of so-called viral load—provides a more direct assessment of viral replication and the antiviral activity of therapeutic agents. Viral load measurements can potentially be applied in two different settings in the evaluation of experimental therapies. First, measurement of viral load can provide a direct pharmacodynamic parameter to evaluate the activity of antiviral drugs, in vivo. Second, viral load measurements have the potential to provide a useful and valid surrogate marker for clinical end points, if it can be rigorously shown that changes in viral load consistently correlate with and anticipate such end points.

Historically, viral load has been measured by quantifying viral antigen (p24) in serum or plasma, sometimes with procedures to dissociate antigen from immune complexes (ICD p24), or by quantifying infectious virus in plasma or peripheral blood mononuclear cells [reviewed in (5)]. Each of these approaches has theoretical and practical limitations in sensitivity, dynamic range, reproducibility, cost, and/or labor intensiveness that make it less than ideal as a viral load marker. For example, circulating p24 antigen may or may not be virion associated [as defined by the ability to pellet the antigen by ultracentrifugation sufficient to pellet HIV type 1 (HIV-1) virions] and may be variably complexed with antibodies that affect the accuracy of quantification, even when immune complex dissociation procedures are used (6). Furthermore, the sensitivity of current immunoassays for detection of p24 antigen is not optimal. The majority of patients with relatively earlier stage disease may be negative for p24 antigen, even with use of ICD p24 assays (7,8). However, 10 pg/ml of p24, the approximate threshold sensitivity for most commercially available assay kits, corresponds to ~100,000 virions/ml (7–9). Quantitative viral cultures are time and labor intensive and expensive, and their utility is limited by issues of sensitivity, reproducibility, dynamic range, and the fact that the vast majority of circulating virus in plasma (on average, >99.99%) is not readily culturable (7,8).

The exquisite sensitivity of polymerase chain reaction (PCR) methods offers great promise for overcoming the sensitivity limitations that affect traditional quantitative viral load measurements. However, whereas PCR methods provide maximal sensitivity for detection of HIV, intrinsic features of the PCR constrain the use of basic PCR methods for quantitative applications (10,11). Internally controlled PCR methods that employ a synthetic template matched to the target region to be amplified can overcome these inherent limitations on the quantitative application of PCR (10,11). In previous studies, we have shown that quantitative competitive-polymerase chain reaction (QC-PCR), an internally controlled PCR technique, provides sensitive and accurate quantification of HIV-1 DNA and RNA, allowing quantitation of as little as 100 copies/ml of HIV-1 in RNA plasma (7,8,12). Our own studies (7,8) and those of other investigators (13–22) have emphasized quantification of circulating virus-associated RNA in plasma by reverse transcriptase (RT)–PCR–based methods, since levels of virus in plasma should be a direct measure of ongoing viral replication, reflecting virus production and release in all anatomic sites that are in continuity with the plasma compartment, including lymphoreticular tissues (23–25). We have shown that levels of circulating virion-associated HIV RNA in plasma, as assessed by QC-PCR, correlate with disease stage and $CD4^+$ cell count and, in preliminary studies, changed significantly with implementation of antiretroviral treatment (7,8).

Previously, we conducted a Phase I/IIA clinical trial evaluating the safety, tolerability, and antiretroviral activity of a novel pyridinone nonnucleoside inhibitor of the HIV-1 RT, L-697,661 (26,27). That study, which involved 135 patients randomly assigned to receive zidovudine or one of three doses of L-697,661, demonstrated significant antiretroviral activity based on changes in plasma p24 antigen levels in patients treated with zidovudine and the higher dose levels of L-697,661. Several features of this clinical trial make it ideal as the basis for the kind of comparative analysis of different viral load measurement methods we report in the present

study. First, the trial was prospective, with subjects blindly randomly assigned to one of four treatment arms. Second, subjects represented a range of disease severity, with $CD4^+$ T cell counts from <10 to 500/mm^3. Third, the study design incorporated a pretreatment washout of all prior antiretroviral therapies for at least 2 weeks prior to study entry. Subjects were given study drugs for 6 weeks, with intensive blood sampling for viral load monitoring, beginning prior to initiation of treatment and continuing to 1 week following temporary discontinuation of treatment, allowing evaluation of the kinetics of viral load changes following initiation and cessation of treatment. Fourth, blood specimens obtained for viral load monitoring were processed in a single laboratory at the clinical trial site, within 4 hours of phlebotomy, with continuous controlled storage at −70°C of replicate plasma aliquots, beginning immediately following processing. These study design features, the demonstrated antiretroviral activity of the agents studied, and the rigorous control over how the specimens were obtained, processed, and stored allowed us to use archived specimens from this study to evaluate the comparative performance characteristics of three different assays of viral load—p24 antigen, ICD p24 antigen, and QC-PCR–determined viral RNA.

SUBJECTS AND METHODS

Patients and Study Design

Specimens analyzed were from two concurrently enrolled clinical protocols (Protocol A, $CD4^+$ T cell counts 200–500/mm^3; Protocol B, $CD4^+$ T cell counts <200/mm^3) conducted at the University of Alabama at Birmingham (27). All patients were assigned to receive one of three oral dosage levels of L-697,661 (50, 300, or 1,000 mg/day, in divided doses) or standard zidovudine therapy (500 mg/day in divided doses). After a washout period of at least 2 weeks, patients were started on assigned therapy for 6 weeks; study treatment was then discontinued for a 1-week washout. Specimens were obtained at weekly intervals for virologic analyses.

To facilitate the interpretation of changes in viral load attributable to changes in treatment, we also collected sequential specimens from three clinically stable HIV-1–infected patients to assess the extent of "natural" variation in QC-PCR–measured viral load. Two of these patients were not receiving treatment, whereas one was receiving stable zidovudine treatment (300 mg/day) over the period studied. Plasma specimens were obtained daily for 5 days, then weekly for a total of 3 weeks. Additional follow-up specimens were obtained from these patients following subsequent changes in treatment regimen.

Specimen Selection

Specimens from all 135 patients in Protocols A and B were collected and processed for viral load assessment by p24 antigen assay, ICD p24 antigen assay, and quantitative plasma virus culture. Specimens from a representative subset of 46 patients (representing the two protocols and all four treatment arms) were selected for QC-PCR evaluation of virion-associated HIV-1 RNA in plasma.

Plasma Viral Load

Plasma specimens were obtained from ACD-A anticoagulated peripheral blood samples, and processed as described in detail elsewhere (7,8), within 4 hours of collection. Aliquots were stored at −70°C until testing. Quantitative plasma virus cultures were performed with fresh specimens, as described (7,8,28), just prior to initiation of treatment (Week 0) and 1 week after completion of the 6-week treatment period (Week 7). Standard and ICD plasma HIV-1 p24 antigen levels were determined at the University of Alabama at Birmingham, with use of commercially available assays according to manufacturer's instructions (Coulter), with batched analysis of all longitudinal specimens for a given patient within the same assay. A threshold cutoff of ≥30 pg/ml was used for both the standard and ICD p24 antigen assays in order to evaluate meaningful changes from baseline associated with treatment. Blinded QC-PCR analysis for quantification of virion-associated HIV-1 RNA was performed at Genelabs Technologies, as described in detail elsewhere (7,8), by operators unaware of the protocol or treatment assignments of the patients whose specimens were being assayed.

Statistical Analyses

The comparability of the baseline characteristics of different treatment groups, the total patient group, and the subset selected for QC-PCR analysis were assessed by analysis of variance. For assessment of treatment effects, plasma viral load results (QC-PCR–determined HIV-1 RNA, p24 antigen, ICD p24 antigen) were normalized as percentage change from pretreatment baseline values for each individual patient, at each sampling point. Mean percentage change from baseline values for each treatment group was determined, and statistical significance of changes from baseline assessed by Wilcoxon's signed rank test. To assess maximal treatment effects irrespective of kinetics, we also calculated the mean maximal decrease on treatment (nadir values), regardless of the time point at which this maximum decrease was noted. All statistical evaluations were performed with use of the SAS statistical analysis software package (SAS Institute).

RESULTS

Baseline $CD4^+$ T cell levels and viral load determinations for the patients studied are summarized in Table 1. With use of the standard p24 assay, 42% of the patients tested had antigen levels of ≥30 pg/ml (mean, 153 pg/ml). Use of the ICD p24 assay increased the number of patients with antigen levels ≥30 pg/ml to 56% (mean, 281 pg/ml). Baseline plasma samples from all 135 patients were tested for culturable virus; 67 (50%) were positive, with a mean infectious titer of 62 tissue culture infectious dose (TCID)/ml. A higher frequency of positive re-

TABLE 1. *Baseline viral load and CD4$^+$ T cell count results*

Protocol (treatment)	CD4 count (cells/μl)	HIV-1 RNA (copies/ml ×10^{-3})	p24 Ag (pg/ml)	ICD p24 Ag (pg/ml)	Plasma culture (TCID/ml)
I. All patients					
Protocol A + B					
(All; n = 135)					
Mean ± SD	195 ± 162	NA	153 ± 111	281 ± 274	62 ± 12
Range[a]	4–683		32–511	30–959	<5–15,625
Number studied	129		130	128	135
% Positive	—		42	56	50
Protocol A					
(All; n = 68)					
Mean ± SD	318 ± 137	NA	150 ± 106	287 ± 300	61 ± 9
Range[a]	(68–683)		34–411	30–959	<5–15,625
Number studied	66		66	66	68
% Positive	—		23	38	29
Protocol B					
(All; n = 67)					
Mean ± SD	71 ± 57	NA	156 ± 113	275 ± 261	63 ± 12
Range[a]	4–211		32–511	36–954	<5–3,125
Number studied	63		64	62	67
% Positive	—		61	76	70
II. Patients studied by QC-PCR					
Protocol A + B					
(All; n = 46)					
Mean ± SD	180 ± 131	187 ± 3.2	136 ± 106	289 ± 253	46 ± 9
Range[a]	3–533	8.6–2,420	34–412	34–859	<5–15,625
Number studied	43	46	46	46	46
% Positive	—	100	43	63	59
Protocol A					
(All; n = 23)					
Mean ± SD	278 ± 109	128 ± 4.0	159 ± 144	317 ± 286	45 ± 12
Range[a]	82–533	8.6–2,420	34–412	34–859	<5–15,625
Number studied	21	23	23	23	23
% Positive	—	100	35	52	48
Protocol B					
(All; n = 23)					
Mean ± SD	82 ± 65	273 ± 2.1	112 ± 69	261 ± 234	46 ± 7
Range[a]	3–241	36.9–739	38–226	43–823	<5–3,125
Number studied	22	23	23	23	23
% Positive	—	100	48	74	70

Baseline viral load and CD4$^+$ T cell results for all patients enrolled in Protocols A and B, irrespective of whether quantitative competitive–polymerase chain reaction (QC-PCR) analysis was performed and all patients for whom QC-PCR viral load analysis was performed. Values shown are geometric means ± SD. HIV-1, human immunodeficiency virus type 1; Ag, antigen; ICD, immune complex–dissociated; TCID, tissue culture infectious dose.

[a] As per the inclusion criteria of the study, all patients had CD4$^+$ T cell counts within specified limits at initial screening; values outside these limits reflect changes in determinations between screening and baseline blood draws.

sults for p24, ICD p24, and plasma viral culture determinations was seen in patients from Protocol B (CD4 cell count <200/mm^3) compared with patients from Protocol A (CD4 cell count 200–500/mm^3), although the mean values for patients with positive results were comparable between the two protocols. In contrast to p24, ICD p24, and plasma virus culture measurements, QC-PCR determinations allowed quantification of viral load in all 46 patients studied by this method. Analysis of variance showed that baseline counts of CD4$^+$ cells and virologic parameters were comparable for the larger group of 135 patients and the representative subset of patients selected for QC-PCR analysis of viral load.

Figure 1 shows p24 and ICD p24 values over the study for those patients with baseline antigen levels ≥30 pg/ml. Baseline counts of CD4$^+$ cells and virologic parameters were not significantly different between treatment groups. Patients treated with zidovudine showed prompt decreases from baseline in p24 antigen levels within 1 week of initiating

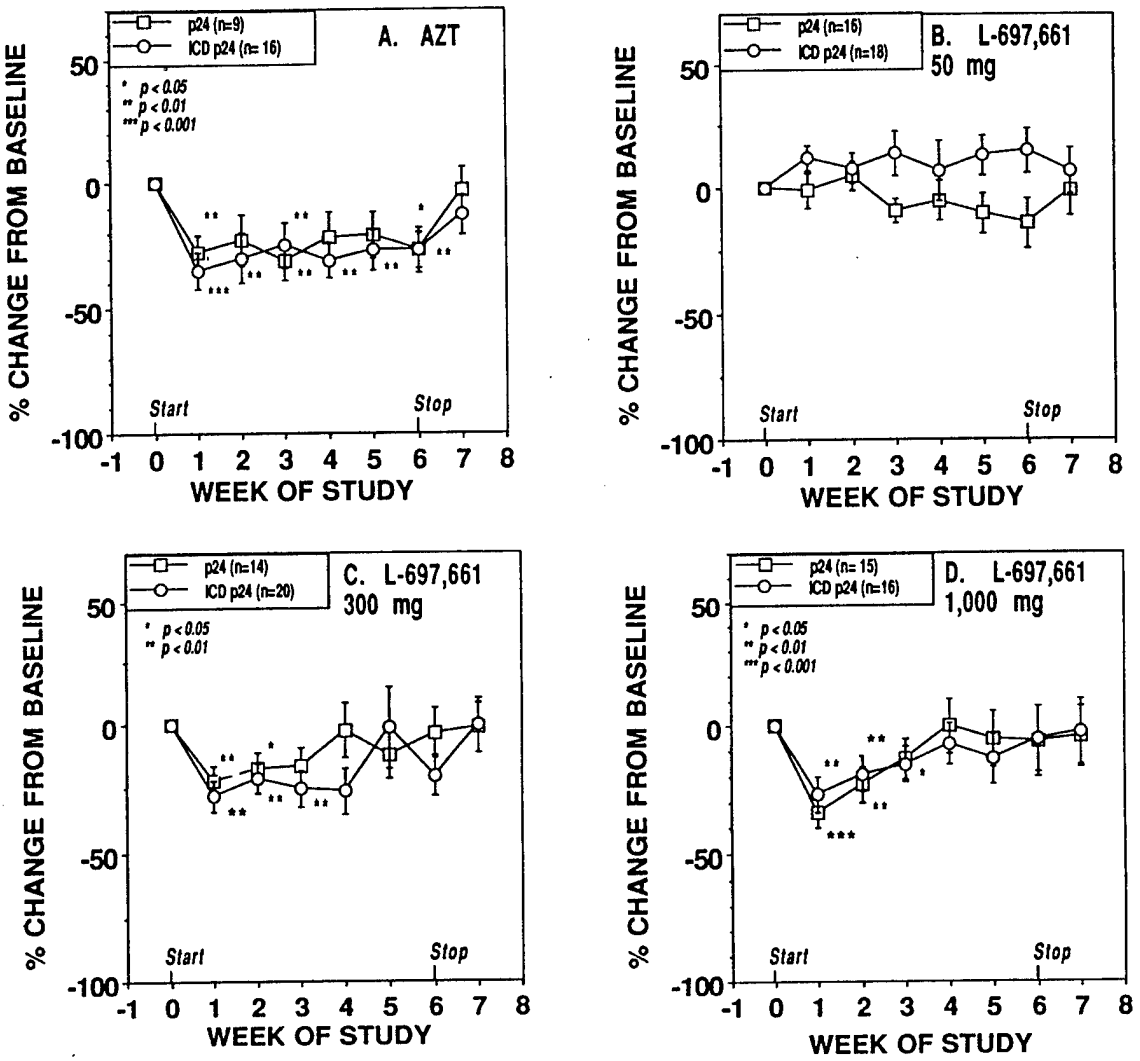


FIG. 1. Changes in viral load measured by p24 and immune complex–dissociated (ICD) p24 assays for all patients enrolled in Protocols A and B, irrespective of whether quantitative competitive–polymerase chain reaction (QC-PCR) analysis was performed. Changes in plasma viral load were measured by p24 antigen assay, and ICD p24 antigen assay in patients undergoing treatment with **(A)** standard zidovudine therapy (AZT) or three different daily dose levels of L-697,661 (**B:** 50 mg, **C:** 300 mg, or **D:** 1,000 mg). Results shown for p24 and ICD p24 are data for the indicated numbers of patients in each group who had ≥30 pg/ml human immunodeficiency virus (HIV) p24 antigen at baseline (Week 0). After a 2-week washout of previous treatment, protocol treatment was started at Week 0, after obtaining baseline viral load specimens. Treatment continued for 6 weeks, then was temporarily stopped for 1 week. Results shown are the mean percentage changes from baseline levels for each parameter ± SEM. p-values are for comparisons with baseline values, by Wilcoxon's signed rank test.

treatment (mean decrease ± SD: 28 ± 20%; p = 0.008), maintained decreased antigen levels for the 6-week duration of treatment, and then experienced a rebound to baseline levels within 1 week of discontinuation of treatment (Fig. 1A). The maximum mean decreases seen in p24 and ICD p24 antigen levels while the patients were receiving zidovudine treatment were 36% (p = 0.004) and 49% (p = 0.0001), respectively (mean of on-treatment nadir values for individual patients). Whereas use of the ICD p24 assay allowed more patients to be monitored (52% vs. 27%), the responses to treatment as measured by the two antigen assays were virtually indistinguishable (Fig. 1A).

Patients treated with L-697,661 showed a trend for dose-related decreases from baseline in circulating p24 antigen levels, as measured by both standard and ICD assay procedures. At Week 1, the percentage decrease in p24 antigen was 1 ± 26% (NS), 22 ± 22% (p = 0.004), and 34 ± 24% (p = 0.0001) for the 50-, 300-, and 1,000-mg/day L-697,661 treatment groups, respectively. With use

of the ICD p24 assay, similar respective decreases of 12 ± 23% (NS), 28 ± 25% ($p = 0.001$), and 27 ± 28% ($p = 0.002$) were noted. In marked contrast to the sustained antigen responses seen in patients treated with zidovudine, p24 antigen had returned to essentially pretreatment levels by Week 6 in L-697,661–treated subjects, despite continuing treatment, with no further rebound occurring when treatment was discontinued (Fig. 1B–D).

Quantitative plasma virus cultures were performed at Week 0 and Week 7 (1 week after the discontinuation of antiretroviral therapy). End point titers of virus were not significantly different before versus after treatment in any of the study groups (data not shown).

Figure 2 demonstrates that among the subset of patients monitored by antigen assays and QC-PCR, a prompt decreases in plasma HIV-1 RNA levels were noted upon initiation of zidovudine therapy (Fig. 2A). Decreases seen in p24 and ICD p24 levels among the subset of patients in this cohort who had quantifiable antigen levels at baseline (≥30 pg/ml) were comparable to the treatment-associated changes seen in the larger group of patients (com-

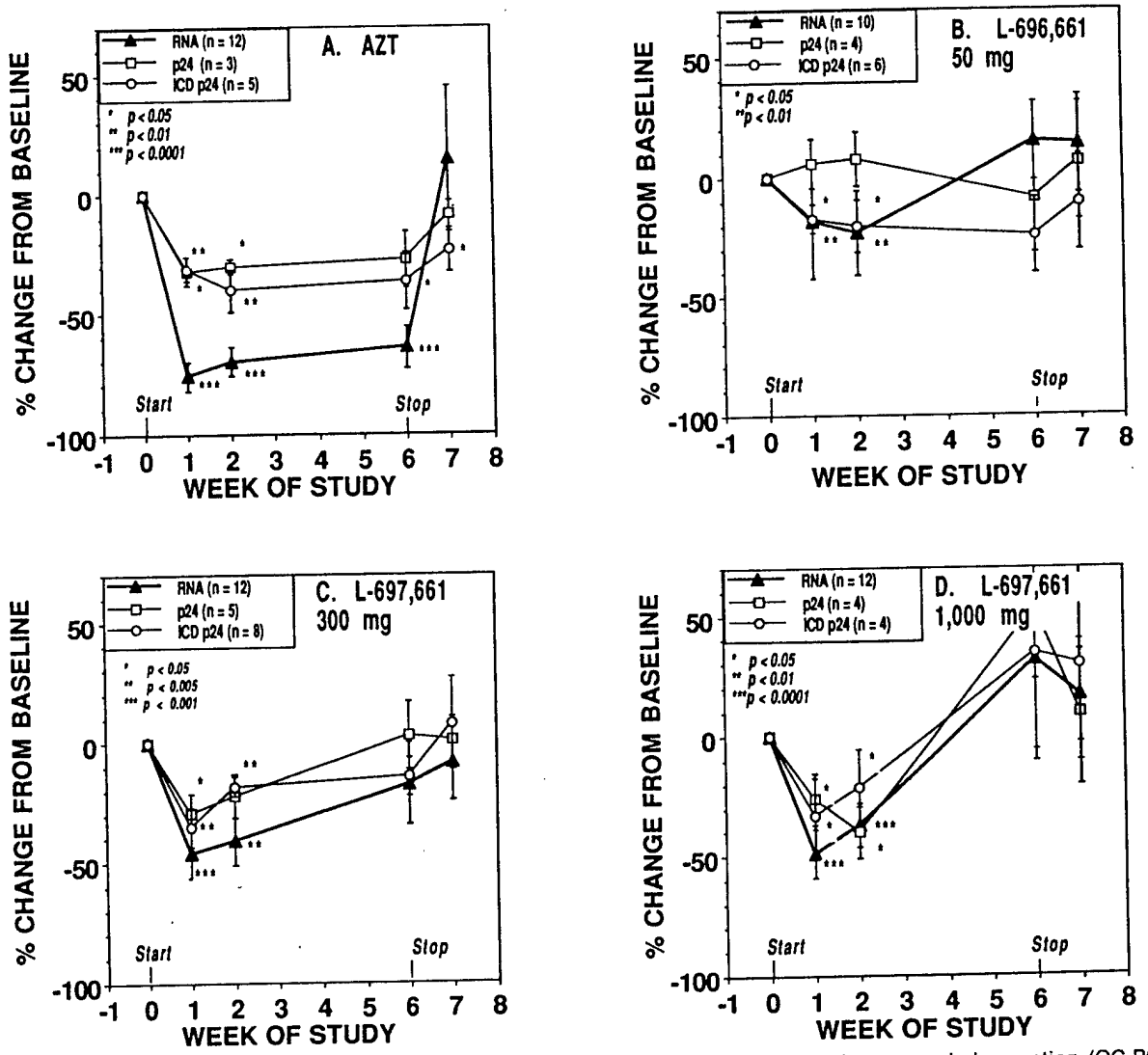
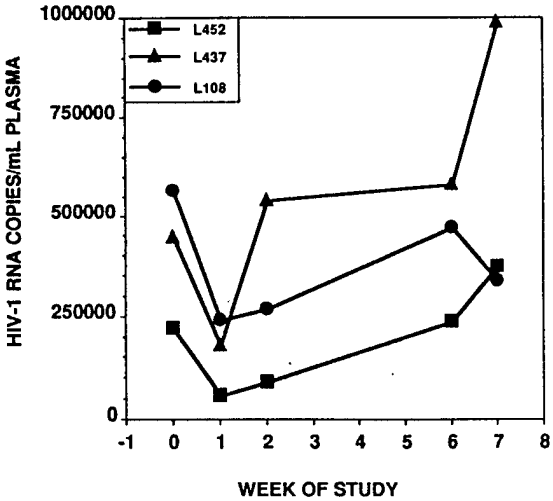


FIG. 2. Changes in plasma viral load for patients studied by quantitative competitive–polymerase chain reaction (QC-PCR). Treatments are as described in the legend to Fig. 1 and in Methods. RNA levels were measurable in all patients. Results shown for p24 and immune complex–dissociated (ICD) p24 are data for the indicated numbers of patients in each group who had ≥30 pg/ml human immunodeficiency virus (HIV) p24 antigen at baseline (Week 0) and represent a subset of the antigen data shown in Fig. 1. Results shown are the mean percentage changes from baseline levels for each parameter ± SEM. p Values are for comparisons at each time point with baseline values, by Wilcoxon's signed rank test.

pare Fig. 1A and 2A). QC-PCR–determined circulating viral RNA levels, however, provided a more sensitive parameter than did the antigen tests, allowing all studied patients to be monitored. In zidovudine–treated patients, within 1 week of starting treatment, QC-PCR–determined viral RNA levels decreased from baseline by 76 ± 22% (SD) ($p < 0.0001$; range 25–95%) compared to 32 ± 11 (NS; range 22–43%) and 31 ± 12 ($p < 0.02$; range 19–47%)) for standard and ICD p24 levels, respectively, in the subset of these patients in whom the antigen parameters could be followed. All of the patients showed at least a twofold decrease in QC-PCR–determined viral load while receiving treatment, with a mean maximum decrease (mean of on-treatment nadir values) of 85 ± 11% (SD) from baseline (range 61–96%). For comparison, none of three clinically stable infected patients (not clinical trial participants) who were intensively studied by QC-PCR for short-term variation in viral load showed a twofold change from the mean value over a 3-week interval of monitoring (data not shown). For the zidovudine-treated group as a whole, decreases in viral load were sustained for the duration of treatment, with rebound of circulating virus to pretreatment levels seen within 1 week of discontinuation of treatment (Fig. 2A).

Among those L-697,661–treated patients in the QC-PCR–monitored cohort in whom antigen levels were ≥30 pg/ml at baseline, both standard and ICD p24 assays showed results similar to those observed in the larger patient group (compare antigen results shown in Fig. 1B–D and 2B–D). QC-PCR also demonstrated treatment-related decreases in viral load within 1 week of initiation of treatment with L-697,661 (Fig. 2B–D). At 1,000 mg/day, the highest dose tested, the decrease in plasma HIV-1 RNA levels at 1 week was 49 ± 36% (SD) ($p < 0.0001$; range 11% increase to 94% decrease). As in the zidovudine-treated group, only QC-PCR allowed all patients to be monitored for viral load over the duration of the study. In striking contrast with the results for the zidovudine group, initial decreases in viral load while receiving treatment were not sustained for the duration of treatment. Instead, levels of circulating virus returned to essentially pretreatment values by Week 6, despite continuing treatment, with no further rebound seen at Week 7, after discontinuation of treatment.

Figure 3 shows illustrative QC-PCR determined viral load results for individual patients treated with L-697,661, along with previously reported data on phenotypic and genotypic resistance to the drug in these patients (27,29). All of the patients shown experienced an initial decrease in plasma viral load while receiving treatment, with a rebound in levels of circulating virus, despite continuing treatment. As has been reported in detail elsewhere (27), com-



Patient	IC90 (nM) Week 0	IC90 (nM) Week 7	RT mutations Week 0	RT mutations Week 7
L-697,661 1,000 mg/day				
L452	50	≥12,000	None	Y181C
L437	50	≥12,000	None	Y181C
L108	400	≥12,000	None	Y181C

FIG. 3. Changes in viral load measured by quantitative competitive–polymerase chain reaction (QC-PCR) for individual patients in response to treatment with L-697-661, 1,000 mg/day. Accompanying tabulated results show available data on phenotypic and genotypic resistance to L-697-661 before treatment (Week 0) and after 6 weeks of receiving treatment (Week 7). IC_{90} denotes the drug concentration required to achieve 90% inhibition of viral replication in vitro, as described (27). The presence of mutations in the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) gene characteristically associated with resistance to nonnucleoside RT inhibitors is indicated in single letter amino acid code, (i.e., Y181C indicates the tyrosine residue at position 181 has been mutated to a cysteine residue (29). [Drug resistance results previously reported in reference (27)].

parison of the RT gene sequences of peripheral blood mononuclear cell–derived virus obtained prior to and following treatment (Week 0 and Week 7) showed the emergence of a characteristic mutation associated with resistance to L-697,661 (Y181C), whereas in vitro drug sensitivity cultures to evaluate phenotypic resistance showed the emergence of high-level viral resistance to L-697,661 [90% inhibitory concentration (IC_{90}) Week 0 = 50–400 nm; IC_{90} Week 7 $\geq$12,000 nm].

DISCUSSION

We conducted a blinded evaluation of the comparative utility of plasma viral load measurements as a means of assessing the activity of antiretroviral agents. Plasma viral load was measured by three alternative methods in stored archival specimens from a prospective, controlled clinical trial. Design features of the clinical protocol and the details of specimen handling and testing made this study extremely well suited for the comparative evaluation of viral load monitoring techniques.

Assessment of levels of circulating p24 antigen has been utilized in many other studies to evaluate the in vivo activity of antiretroviral therapy [30,31, reviewed in (5)]. However, the limited sensitivity and dynamic range of the procedure have restricted its use to the subset of patients with high viral loads in whom antigen levels can be followed. In the current study, we utilized a conservative lower limit of 30 pg/ml for both p24 and ICD p24 assays, taking into account both the reproducibility and threshold sensitivity of these assays, in an attempt to ensure that percentage changes from baseline would represent real changes when data were normalized. As expected, the use of the ICD p24 antigen assay increased the fraction of patients that could be evaluated (from 42 to 57%), in general agreement with other reports (6). Thus, with use of procedures currently recommended by commercial suppliers for acidification to facilitate dissociation of immune complexes and a threshold cutoff of 30 pg/ml, ~15% more patients could be monitored using the ICD p24 procedure, compared with the standard p24 assay. Results for p24 and ICD p24 were highly correlated in this study (Figs. 1 and 2) and others (7,8). However, despite the greater sensitivity of the ICD p24 assay, the extent of treatment-related change in viral load observed was not significantly different from the standard p24 antigen results. Zidovudine treatment resulted in a sustained reduction in viral burden, as measured by both standard and ICD p24 assays, throughout the 6-week duration of zidovudine treatment. In contrast, patients treated with L-697,661 exhibited only transient reductions in antigen levels, which returned to baseline within the 6-week treatment period, apparently reflecting the rapid emergence of drug-resistant virus.

In this analysis of patients with $CD4^+$ T cell counts $<500/mm^3$, all 46 subjects studied by QC-PCR had quantifiable virion-associated viral RNA in their plasma at baseline, including the subset of these patients in whom plasma levels of HIV-1 p24 antigen were not quantifiable with use of either standard or ICD p24 assays. The incremental sensitivity of QC-PCR would be even more important in monitoring patients with less-advanced HIV disease ($CD4 > 500/mm^3$), in whom quantifiable levels p24 antigen and other viral load markers are present at even lower levels (7,8). QC-PCR demonstrated prompt, sustained decreases in plasma viral load with initiation of zidovudine therapy, with rapid rebound of circulating virus within 1 week of discontinuation of treatment. Virologic responses were seen upon re-initiation of zidovudine following temporary discontinuation, even in patients with nearly 3 years of prior zidovudine treatment. The overall viral load response measured by QC-PCR was of greater magnitude than that observed with measurements of p24 antigen using standard and ICD approaches.

QC-PCR analysis also documented treatment-associated, dose-related decreases in plasma viral load in patients treated with L-697,661; antigen responses showed similar trends, where measurable, in these patients. However, in sharp contrast to patients treated with zidovudine, for patients treated with L-697,661, after initial decreases, levels of virus measured by all methods rebounded to pretreatment levels by Week 6, despite continuing treatment (Fig. 2). On-treatment rebound in this group was associated with the ability to isolate virus resistant to L-697,661 from patient peripheral blood mononuclear cells at Week 7, after the conclusion of the posttreatment washout period (Fig. 3).

The use of QC-PCR and batched standard and ICD p24 assays in this study provide a more comprehensive analysis than has previously been available in standard clinical research evaluations and yield additional insights into the virologic response to antiretroviral therapy. Based on real-time (nonbatched) analysis using a standard p24 antigen assay from a different manufacturer (Abbott Labora-

tories), we previously reported statistically significant, transient decreases in antigen levels in patients treated with L-697,661 (27). These data, along with results of viral drug sensitivity studies, led to the conclusion that drug resistance had developed. The L-697,661 study medication was discontinued and the study terminated.

Whereas qualitatively similar, the present results provide a more comprehensive view of the virologic response to treatment in this clinical study. Sustained, statistically significant decreases in p24, ICD p24, and viral RNA levels were seen in zidovudine-treated subjects (Figs. 1 and 2). Transient, dose-related changes in these parameters were seen in L-697,661–treated patients, although the maximal effect seen in L-697,661 patients was less than that seen in the zidovudine-treated group (Figs. 1 and 2). We interpret the seemingly limited virologic effect of L-697,661 treatment to have resulted primarily from the rapid appearance of drug-resistant virus (27) (Fig. 3), although less than optimal drug levels in vivo may have also contributed. In recent studies, we have also documented transient changes in viral load, measured by standard and ICD p24 antigen assays, QC-PCR, and other methods, in patients treated with other nonnucleoside RT inhibitors. These changes have occurred with similar kinetics and also appear to be associated with the development of drug-resistant virus (G. M. Shaw et al., unpublished observations).

These results clearly indicate the utility of QC-PCR monitoring of viral load for assessment of antiviral activity of antiretroviral therapy in vivo. Consistent with recent reports (32,33), we found that in clinically stable patients plasma HIV-1 RNA viral load is a relatively stable parameter and that measured variation attributable to spontaneous short-term fluctuations in actual levels of circulating virus, compounded by assay variability [QC-PCR interassay coefficient of variance (c.v.) = 22%, (7)], are modest compared to changes that can be seen with active treatment. In the absence of changes in treatment, viral load measured over a period of 3 weeks in three different patients not participating in the clinical trial was stable, with maximum variation from the mean value over this period of less than twofold. In contrast, marked changes in RNA levels were seen following changes in treatment in two of these patients. In one, initiation of dideoxycytosine therapy (2.25 mg/day) led to a 19-fold decrease in plasma HIV-1 RNA level, whereas in the other, temporary withdrawal of zidovudine resulted in an increase of nearly ninefold, from the respective mean values over the 3 weeks of monitoring prior to the change in treatment (data not shown).

In following changes in viral load in patients treated with zidovudine or L-697,661 under clinical trial conditions, QC-PCR measurements tracked along with tests for p24 antigen, using standard and immune complex–dissociated assay methods, but were far more sensitive and dynamic, allowing monitoring of viral load in patients who could otherwise not be followed for virologic responses to treatment. In principle, since each virion would be expected to contain a fixed number of p24 molecules and two RNA copies (discounting "empty" particles), these two parameters might be expected to move exactly in parallel. Indeed, in previous studies (7,8) and in the present results, there was a good, but not absolute, correlation between p24 antigen and RNA levels. In the current studies, the less dynamic changes in the antigen tests compared with the QC-PCR results may be related to the variable proportion of p24 antigen that is virion associated in different plasma specimens (G. M. Shaw et al., unpublished observations). Nonvirion-associated p24 antigen may represent a pool that does not turn over as rapidly in response to treatment-related changes in levels of viral replication.

The present study involved the blinded, retrospective analysis of archived specimens and indicates the utility of QC-PCR for tracking treatment-related changes in circulating levels of virus. Additional studies in which QC-PCR has been used prospectively for assessment of treatment-associated changes in viral load suggest that the method may be useful in this context as well and that baseline levels of circulating viral RNA may be a useful parameter for stratification for data analysis or may provide a useful study entry criterion (J. Kahn, et al., unpublished observations).

Further studies will be required to determine whether QC-PCR and several other viral load measurement techniques based on quantification of virion-associated HIV-1 RNA in plasma (13–22,34,35) will usefully serve as valid surrogate markers that predict eventual clinical responses in the evaluation of the efficacy of experimental antiretroviral therapies, complementing existing methods for monitoring HIV-1–infected patients. If this promise can be fulfilled, it would represent a significant advance in facilitating the rapid and efficient evaluation of new treatments.

Acknowledgment: This work was supported in part by grants AI35467 (J.D.L.) and AI32775 (M.S.S.) from the National Institutes of Health, by grant DAMD 17-93-C-3146 (GMS) from the Department of Defense, and by the core research facilities of the University of Alabama at Birmingham, Center for AIDS Research, the Birmingham Veterans Administration Medical Center, and the General Clinical Research Center, University of Alabama at Birmingham. The authors thank Drs. L. Corey, R. Coombs, and D. Kuritzkes for helpful discussions, and R. Cuevas for assistance with preparation of the manuscript.

Informed consent was obtained from all patients who participated in this study, in accordance with the procedures of the human subjects review board of the University of Alabama at Birmingham.

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HIV viral load markers in clinical practice

Plasma HIV RNA determinations are an important prognostic marker of disease progression and, when used appropriately, provide a valuable tool for the management of individual patients. But what constitutes appropriate use?

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The development of new molecular techniques designed to detect circulating virion-associated HIV RNA in plasma has created an opportunity to study viral dynamics and HIV pathogenesis in substantial detail. Prior beliefs, based on the concept of a prolonged phase of relative virologic latency in the period before symptoms become evident have been replaced by a new paradigm of ongoing, high-level viral replication from the time of initial infection until death (Fig. 1). Indeed, as many as 10 billion new HIV virions are produced per day, with a half-life in plasma of 6 hours[1,2,3]. $CD4^+$ lymphocytes, one of the principal cell targets responsible for viral replication *in vivo*, are also produced in high numbers and, once productively infected, have a half-life of about 1.6 days. The life-cycle of the virus, from infection of one cell to the production of new progeny, which infects the next cell, is 2.6 days[3]. This extraordinarily high level of viral replication, cell destruction and cell replacement has led to a dramatic shift in clinical management of HIV-infected patients, and, in particular, the use of antiretroviral therapy.

Before the development of these new molecular techniques, quantitative culture of peripheral blood mononuclear cells (PBMCs) or plasma was used to estimate the infectious titer of HIV in the blood[4,5]. Increasing plasma virus titers were associated with clinical progression while decreases in plasma virus accompanied treatment with active drugs. However, fewer than 50% of patients with $CD4^+$ counts greater than 200 cells/μl had positive plasma cultures, and inherent biologic variability in virus quantitation required that a 25-fold (approximately 1.4 log) increase was seen before it was likely to be clinically meaningful. In contrast, HIV RNA detection techniques revealed measurable virus in the plasma of virtually all HIV-infected patients regardless of clinical stage[6]. Moreover, plasma RNA levels exhibited a wide dynamic range, correlated significantly with clinical stage, and fell rapidly following the initiation of effective antiretroviral therapy. Despite these promising attributes, it was not known whether plasma virus levels could be accurately and reproducibly measured in a clinical setting or whether they would be predictive of clinical outcome. Recent findings indicate that plasma HIV RNA determinations are an important prognostic marker of disease progression and provide a valuable tool for the management of individual patients.

The assays

Three commercially available plasma HIV RNA assays — branched DNA (bDNA), RT-PCR, and Nucleic Acid Sequence-Based Amplification (NASBA) — have not yet been approved by the US Food and Drug Administration for use in routine patient management. The bDNA technique amplifies the signal from a captured viral RNA target by sequential oligonucleotide hybridization steps while RT-PCR and NASBA use enzymatic methods to amplify target HIV RNA into measurable amounts of nucleic acid product[7,8,9]. Target RNA sequences in plasma are quantified by comparison with internal or external reference standards, depending on the assay used. Despite the differences in methodologic approach, plasma HIV RNA measurements obtained with the three assays are strongly correlated ($R \sim 0.90$)[10,11] and each has low intra-assay sample variability (approximately 0.12 to 0.2 log on repeated testing of single samples)[10–12] (Table 1). Plasma HIV RNA levels are relatively stable on a week-to-week or month-to-month basis in clinically stable patients, as long as antiretroviral therapy is not instituted or changed (biologic variability ~0.3 log)[13]. Therefore, sustained changes in the plasma HIV RNA levels of >0.5 log (that is, greater than threefold) generally reflect biologically relevant changes in the level of viral replication.

Correlation of HIV RNA levels to stage of disease

Early studies demonstrated a clear association between the titer of culturable virus in the plasma and the clinical stage of disease[4,5,14]. In patients with the acute seroconversion syndrome and those with advanced HIV disease (<100 $CD4^+$ lymphocytes/μl), plasma viral titers were generally in the range of 100 to 10,000 tissue culture infectious doses (TCID)/ml while in asymptomatic patients with higher $CD4^+$ lymphocyte counts (>300 cells/μl) plasma titers were low or non-detectable. The use of quantitative HIV RNA techniques has further defined the association between plasma virus levels and clinical disease sta-

Generalized virologic and immunologic course of HIV disease

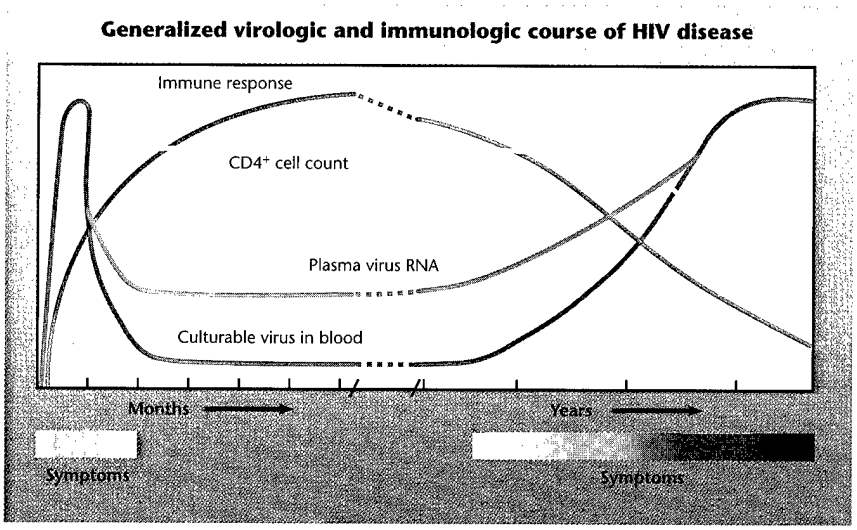

Fig. 1 Schematic representation of natural history of HIV disease.

tus. Most importantly, in virtually all untreated patients, viral RNA is detectable in plasma regardless of clinical stage[6,10,15]. Plasma HIV RNA levels are highest in acute (primary) infection (former CDC stage I) and late-stage disease (CDC IV) and are intermediate in titer in earlier clinical stages (CDC II and III). The relative correlation between plasma virus titer (by culture methods) and quantitative PCR values is shown in Fig. 2.

Table 1 Characteristics of Plasma HIV RNA Assays

Assay (manufacturer)	Linear Dynamic range (copies/ml)	Observed intra-assay standard deviation range (log10)[a]	Preferred anticoagulant
RT-PCR *(Roche Molecular Systems)*	$10^{2.6}$–$10^{5.9}$	<0.15–0.33	ACD/EDTA
bDNA *(Chiron)*	1×10^4–1.6×10^6	0.12–0.2	EDTA
NASBA *(Organon Teknika)*	4×10^2–4×10^7	0.13–0.23	ACD/EDTA/HEP

Higher values can be measured with dilution of the specimen into the linear dynamic range for each assay. [a]Ranges are representative of an ongoing HIV RNA certification program sponsored by the National Institutes of Health, Division of AIDS, Virology Quality Assurance Program[12]. ACD = acid citrate dextran (citrate; yellow-top tube) EDTA = ethylenediaminetetraacetic acid (purple-top tube). HEP = heparin (green-top tube).

Ability of plasma viral RNA to predict clinical outcome

$CD4^+$ lymphocyte counts have been viewed as the best predictor of the risk of developing AIDS-related complications. The risk of developing HIV disease or dying over the next 24 months is <5% among individuals with $CD4^+$ counts above 500 cells/μl and >70% among those having fewer than 50 cells/μl (ref. 16). Despite its value as a general marker of disease stage, the $CD4^+$ count alone is inadequate as a means of measuring prognosis and response to antiretroviral therapy. $CD4^+$ counts are subject to substantial biologic variability and exhibit a limited dynamic range (approximately 2 log)[17,18]. Most importantly, decreases in $CD4^+$ cell counts occur as a result of viral replication and, in that sense, represent a clinical endpoint rather than a "surrogate marker" of disease activity. It is this very process — HIV-mediated lymphocyte destruction — that physicians attempt to prevent rather than observe.

Higher HIV RNA levels correlate with lower baseline $CD4^+$ counts, more-rapid declines in $CD4^+$ counts, and more rapid disease progression[19–23]. Patients with >100,000 HIV RNA copies/ml of plasma within six months of seroconversion were tenfold more likely to progress to AIDS over five years than were those with <100,000 copies/ml (ref. 22). Patients who consistently maintained an HIV RNA copy number of less than 10,000/ml did not progress to AIDS during the next five years; HIV RNA levels tended to increase among progressors. Thus, maintenance of plasma HIV RNA levels below 10,000/ml in early HIV disease appears to be associated with decreased risk of progression to AIDS. However, in patients with more advanced disease (median $CD4^+$ cell count, 89/μl), disease progression occurred in up to 30% of patients with fewer than 10,000 HIV RNA copies/ml (ref. 20, 21).

In a recent study, a single determination of plasma viral RNA in 181 seropositive individuals provided important prognostic information concerning time to AIDS and death[23]. Subjects were stratified by plasma HIV RNA level (quartiles of <4,530; 4,531–13,020; 13,021–36,270; and >36,271 HIV-1 RNA copies/ml plasma) and followed for as long as 11.2 years. Substantial increases in disease progression rates were associated with higher baseline viral levels. For patients with $CD4^+$ cell counts above 500/μl (median, 780/μl), >70% progressed to AIDS and died within 10 years if their baseline RNA level was >10,200; in contrast, <30% of those with <10,200 copies/ml died within 10 years. A threefold higher baseline HIV RNA level was predictive of a 60% increased hazard of death. A single plasma virus RNA determination predicted clinical events oc-

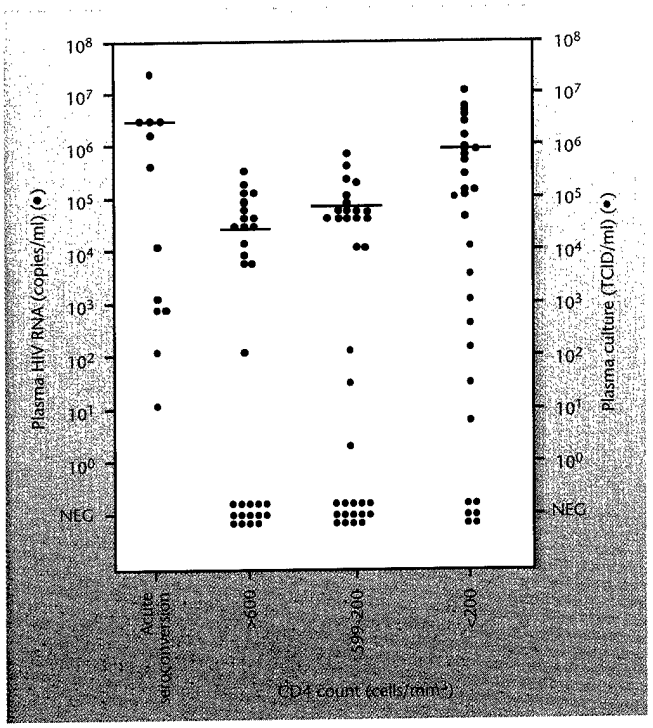

Fig. 2 Comparison of plasma HIV levels as measured by infectious titer in tissue culture (tissue culture infective dose/ml (TCID/ml); blue circles) versus amplified genomic RNA via QC-PCR (copies/ml; red circles) in patients at different stages of HIV disease. Mean values of HIV RNA levels are indicated by horizontal bars. All values obtained for each assay were determined from paired specimens, as reported in Piatak *et al.*[6] The observed discrepancy between total virus levels determined by direct RNA measurements and those determined by culture (generally 100–10,000 to 1) is typical of retroviruses, which are known to exhibit high frequencies of genetic and phenotypic defectiveness. The fact that direct bDNA, RT-PCR and NASBA methods detect primarily virus that is non-culturable is not relevant to their clinical utility since plasma virus, infectious or not, is a direct measure of virus production and the processes sustaining HIV infection and pathogenesis.

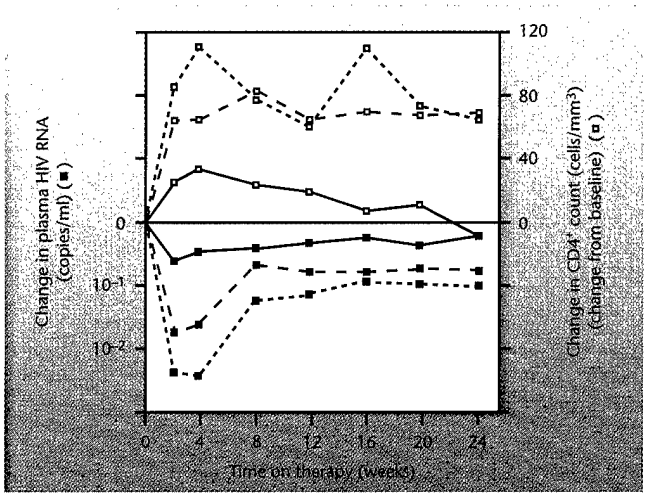

Fig. 3 Composite of plasma HIV RNA and CD4+ cell count responses among a group of patients receiving three different antiretroviral regimens: zidovudine monotherapy (ZDV, 600 mg/day; solid lines), zidovudine plus lamivudine (ZDV/3TC, 600 mg/day, 300 mg/day, respectively; long-dashed lines), and the protease inhibitor, indinavir (MK-639, 2400 mg/day; short-dashed lines). All patients were naive to their respective treatment regimens. For each treatment group, the relative HIV RNA and CD4+ count treatment responses appear inversely proportional, although individual exceptions to this association exist.

curring as long as 10 years later, similar to predictions determined through the staging of certain malignancies (for example, Hodgkin's disease). Placed in the context of clinical practice, plasma HIV RNA levels appear to be the best predictor of long-term clinical outcome while CD4+ lymphocyte counts remain the best predictor of immediate or short-term risk of developing a new opportunistic disease process.

Response of plasma HIV RNA to antiretroviral therapy

Plasma HIV RNA measurements are useful for rapidly evaluating the relative antiretroviral effect of new or available drugs or regimens in clinical trials[1,19,24,25]. Effective antiretroviral therapy significantly decreases HIV RNA levels in plasma within one week of the start of treatment. No significant decrease in the plasma levels within this period suggests that the regimen has no antiretroviral activity. Zidovudine monotherapy results in a median 0.7 log decrease in the plasma HIV RNA level within two weeks, which returns toward baseline values by 24 weeks[19,20]. Lower HIV RNA troughs (~1.5 log decreases) and higher CD4+ lymphocyte peaks are observed with nucleoside combinations and the responses are generally more durable, often persisting for more than one year[26] (Fig. 3). Protease inhibitors in combination with nucleoside therapy results in dramatic and sustained reductions in plasma viral RNA (~2.0 log) and, in one trial, treatment-associated reductions were associated with a survival benefit in patients with advanced disease[1,2,27–29]. Decreases in plasma HIV RNA levels generally correlate with increases in the CD4+ lymphocyte count in patients in whom effective antiretroviral therapy is initiated. In patients in whom plasma HIV RNA levels initially decline but return to pretreatment values, the loss of antiviral effect has been associated with the emergence of drug-resistant strains of HIV (ref. 1, 24, 30, 31).

Antiretroviral induced changes in plasma HIV RNA level and CD4+ lymphocyte count are both independent predictors of disease progression. In one study, each twofold (0.3 log) decrease in the HIV RNA level during treatment was correlated with a 27% reduction in the relative hazard of progression[20]. In another study, each three-fold (0.5 log) decrease in HIV RNA level was associated with a 63% reduction in relative hazard of progression ($P = 0.02$)[19]. In a third study, a 1.0 log treatment-induced reduction in HIV RNA was associated with an 80% reduction in relative risk of disease progression and was a more powerful predictor of clinical outcome than CD4+ cell counts or other virologic measures[25]. In general, a decrease in plasma HIV RNA level along with an increase in the CD4+ lymphocyte count explain a significant part of the treatment effect observed in these studies.

The effect of emerging data on clinical practice

As a result of the overlapping nature of clinical research and patient care and the rapid translation of clinical research findings (based on plasma HIV RNA endpoints) to patient management, many clinicians are using HIV RNA assays in their practices. As an example, among 915 HIV clinicians attending one of five International AIDS Society–USA-sponsored, advanced courses on the management of HIV disease, in spring 1995, 20% (172) used HIV RNA measurements in their practice. Yet despite increasing evidence demonstrating the value of viral load determinations, many practitioners are uncertain about the op-

Table 2 Summary of interim recommendations

Parameter	Recommendation
• Plasma HIV RNA level that suggests initiation of treatment	• More than 5,000–10,000 copies/ml and a CD4+ count/clinical status suggestive of progression; >30,000–50,000 regardless of laboratory/clinical status
• Target level of HIV RNA after initiation of treatment	• Undetectable; <5,000 copies/ml is an acceptable target
• Minimal decrease in HIV RNA indicative of antiviral activity	• >0.5 log decrease
• Change in HIV RNA that suggests drug treatment failure	• Return to (or within 0.3 to 0.5 log of) pretreatment value
• Suggested frequency of HIV RNA measurement	• At baseline: 2 measurements, 2–4 weeks apart • Every 3 to 4 months or in conjunction with CD4+ counts • Shorter intervals as critical decision points are neared • 3–4 weeks after initiating/changing therapy

timal use of these tests and are forced to make therapeutic decisions on the basis of anecdotal experience. Because of the need for guidelines to assist practitioners in the use of plasma HIV RNA measurements, the IAS–USA convened an *ad hoc* panel of investigators and clinicians to make recommendations for the use of these assays in clinical practice. The recommendations of the panel were developed in conjunction with a series of questions related to the natural history of HIV disease, current understanding of correlations between plasma HIV RNA levels and prognosis or response to antiretroviral therapy, and the performance characteristics of the assays.

Should plasma HIV RNA levels be used routinely in practice?

Yes. Monitoring plasma HIV RNA levels adds important information for patient management, including information on risk of disease progression, when to initiate therapy, the degree of initial antiretroviral effect achieved and when a drug regimen is failing. Although there are no published data from controlled clinical trials as yet, new data from controlled treatment trials and natural history studies strongly support their use in routine practice. The $CD4^+$ lymphocyte count remains an essential index for making decisions regarding prophylaxis for opportunistic infections and for evaluating the immunologic effects of antiretroviral therapy. Since plasma HIV RNA levels and $CD4^+$ lymphocyte count determinations are independent predictors of clinical outcome, their combined use provides a more complete picture of an individual patient's status and response to therapy.

When should antiretroviral therapy be initiated?

The goals of antiretroviral therapy are to limit or delay disease progression and increase survival. Given the dynamic interaction between viral replication and $CD4^+$ lymphocyte destruction and the results of clinical studies showing increased survival in association with significant reduction in plasma RNA levels, the best way to achieve these goals is by minimizing viral replication. Steady-state plasma viral RNA levels are directly related to rates of virus production in lymphoreticular tissues and changes in plasma viral load can be used to assess antiretroviral drug effects in otherwise inaccessible tissue compartments. Ideally, the goals of therapy are to reduce the plasma HIV RNA level as much as possible and for as long as possible.

Recent disease progression cohort data and clinical trial results showed that there is a continuum of increased risk for AIDS and death as HIV RNA levels increase. Patients with very low HIV RNA levels (for example, <5,000–10,000 copies/ml) have a better clinical prognosis than those individuals with only modest elevations in RNA levels (for example, 10,000–25,000 copies/ml). Some investigators, including the authors, have concluded that plasma HIV RNA levels >30,000–50,000 copies/ml warrant the initiation therapy, regardless of $CD4^+$ cell count or clinical status. In patients with plasma HIV RNA levels >5,000–10,000 copies/ml but less than 30,000, the decision to start therapy should be made in conjunction with $CD4^+$ cell counts and clinical status. If antiretroviral treatment is initiated according to current clinical guidelines (based solely on $CD4^+$ count values), plasma HIV RNA determinations may be helpful for patients who have $CD4^+$ counts near the current threshold values (for example, 500 $CD4^+$ cells/μl). A high viral load in such patients might provide additional impetus for initiation of treatment.

What level of plasma HIV RNA should be sought?

Ideally, undetectable levels of plasma HIV RNA should be sought. However, maximal clinical benefit might be achieved by maintaining plasma HIV RNA levels below 5,000 copies/ml. It has not been shown whether plasma HIV RNA reduced to a particular level by antiretroviral therapy carries the same risk of clinical progression as that same HIV RNA level without antiretroviral therapy; prospective clinical trials are urgently needed to address this question. Because sustained suppression below 5,000 copies/ml may not be achievable for many patients using the currently available therapies, this recommendation represents a reasonable "target" level for some patients, but certainly less than "ideal" in others.

For a patient, what suggests a drug is working?

A three-fold or greater sustained reduction (>0.5 log) of the plasma HIV RNA levels is the minimal response indicative of an antiviral effect, given within-assay variation (~0.15–0.2 log) and natural biologic variation of plasma HIV RNA *in vivo* (~0.3 log). Reductions of this magnitude have been associated with clinical benefit in treatment trials. It is not known whether a reduction in plasma viral RNA of any given magnitude has the same significance in terms of clinical benefit irrespective of the initial pretreatment RNA value. That is, it is not clear whether a 1.0 log (tenfold) reduction in virus load in a patient with a pretreatment level of 1,000,000 copies/ml has the same clinical significance as 1.0 log reduction in a patient with an initial pretreatment level of 10,000 or 100,000 copies/ml. It is likely that the clinical benefits of antiretroviral therapy are related to the duration as well as the magnitude of HIV suppression (that is, the area under the curve), although the precise duration of HIV suppression necessary to result in measurable clinical benefit still needs to be clearly defined.

With existing antiretroviral drug regimens, it is not realistic to expect that lowest plasma HIV RNA levels achieved can be maintained indefinitely. Thus, the return of HIV RNA levels to pretreatment values (or to within 0.3–0.5 log of the pretreatment value), confirmed by at least two measurements, is indicative of drug failure and should prompt consideration of alternative treatment regimens. Decisions to institute changes in therapy should be made using the plasma HIV RNA value in conjunction with CD4+ lymphocyte count and clinical status.

How often should plasma HIV RNA levels be measured?

For the initial determinations of the HIV RNA plasma level, two measurements should be obtained 2–4 weeks apart. Subsequently, we suggest that measurements might be obtained along with the $CD4^+$ lymphocyte count (every 3–4 months, according to current convention), since serial determination of both markers simultaneously provide useful information. Viral load assessments may be made at shorter intervals (for example, every 4 weeks) as critical clinical decision points — such as the return of the viral load level to baseline values — are approached. Ideally, plasma HIV RNA levels should be measured 3–4 weeks after initiating or changing antiretroviral treatment to determine the magnitude of the response. Because of the effects of immune activation on viral load, HIV RNA levels should not be measured within a month of acute illnesses or within a month after influenza and pneumococcus immunizations. Increases in HIV RNA levels in blood of as much as 300-fold have been observed within two weeks of routine immunizations against influenza, tetanus, or pneumo-

coccus[32-33]. These increases are transient, returning to preimmunization levels within four weeks of immunization. Increases are also associated with reactivated genital herpes and tuberculosis, and presumably occur with other acute illnesses.

How should samples be stored, handled and processed?

Optimal procedures for storage, handling, and processing of patient samples have yet to be fully defined. Practitioners should be familiar with the particular assay they have chosen for HIV RNA quantification and the specific sample-handling factors necessary for that assay. Each provider should adopt consistent procedures for handling specimens, including using the same collection tube and anticoagulant, processing techniques, transport and storage procedures, and the same assay for every sample from the same patient. To minimize signal degradation, all plasma specimens should be separated and frozen within 6 hours of collection. If this approach is not possible, the plasma should be removed and refrigerated. Less desirably, the whole blood could be refrigerated, but not for more than 24 hours before separation and freezing are completed.

Plasma HIV RNA quantitation has provided valuable insights into HIV pathogenesis and the activity of antiretroviral regimens. The optimal use of plasma HIV RNA assays will become better understood as more data become available. Of obvious importance is the continued demonstration of clinical benefit in association with treatment strategies that focus on suppressing the viral load as the primary objective of therapy, as is better characterization of the types of events that cause perturbations in plasma HIV RNA levels. Until these issues are further elucidated, these recommendations provide a reference point for the use of plasma HIV RNA as a marker today.

Acknowledgments

The authors and the International AIDS Society–USA are grateful to Margaret Chesney and Donald Chambers for their assistance in development and data analysis of the International AIDS Society–USA survey, to Jane Garrison and Matthew Stenger for editorial assistance, to William Moore and Jennifer B. Wilson for assistance with the figures and to John Mellors for careful review of the manuscript.

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(in press; 12/11/96)

10210-NP

Nature Medicine

December 9, 1996

Antiviral Pressure Exerted by Human Immunodeficiency Virus Type 1-Specific Cytotoxic T Lymphocytes (CTL) During Primary Infection Demonstrated by Rapid Selection of CTL Escape Virus

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Abstract

The development of vaccines and therapeutic strategies to combat human immunodeficiency virus (HIV) infection is hampered by the current incomplete understanding of the contribution made by different immune defense mechanisms to virus control. The HIV-1-specific cytotoxic T lymphocyte (CTL) response is temporally associated with the decline in viremia during primary HIV-1 infection[1,2], but definitive evidence that it has an impact on virus replication *in vivo* is lacking. Here we show that in a patient whose early CTL response was focused on a highly immunodominant epitope in gp160, there was rapid selection for a virus population bearing amino acid changes at a single residue in gp160 which conferred escape from recognition by epitope-specific CTL. The transmitted virus strain was completely eliminated and replaced by CTL escape variants within 136 days of the onset of symptoms. This demonstrates that virus-specific CTL exert substantial pressure on virus replication at early times after HIV-1 infection, comparable to antiretroviral chemotherapy. Although the antiviral CTL response evolved to recognize new epitopes and virus replication was initially contained, this patient subsequently underwent rapid disease progression. One aim of HIV-1 vaccines should thus be to elicit strong CTL responses against multiple codominant viral epitopes in order to maximally suppress virus replication and escape.

Introduction

The development of effective vaccines and immune-based therapeutic strategies to combat human immunodeficiency virus type 1 (HIV-1) infection has been hampered by the lack of clear understanding of the contribution that humoral and cell-mediated immune responses make to virus containment, and also of why the immune response fails to control virus replication more completely in the early stages of the infection. In many other virus infections, the virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) response plays a key role in viral clearance. Likewise, recent studies in animal models suggest that vaccine-induced protection against simian and feline immunodeficiency viruses is correlated with the induction of strong antigen-specific CTL responses[3,4]. HIV-1-specific CTL activity has also been demonstrated in exposed seronegative individuals[5,6], although the relative contributions made by the cell-mediated immune response and genetically-conferred resistance[7] to the lack of infection in these individuals remains unclear. Further suggestive evidence that virus-specific $CD8^+$ CTL make an important contribution to containment of virus replication during infection with HIV-1 comes from the observations that HIV-1-infected long-term nonprogressors have high levels of HIV-1-specific CTL precursors (CTLp)[8,9], whilst HIV-1-specific CTLp are lost during progression to AIDS[8-11]. However the immunosuppressive nature of HIV-1-associated disease makes it difficult to assign cause and effect when a loss of immune function is observed in association with increasing viral titres in the end stages of the infection.

Study of the events taking place early after infection with HIV-1 has shown that virus-specific $CD8^+$ CTL responses are induced prior to seroconversion, and are temporally associated with the fall in viremia during acute infection[1,2]. Whilst these observations are consistent with the antiviral CTL response playing a role in containment of virus replication early after infection, it

has been suggested that the decline in primary viremia may simply be a result of population dynamics, with virus replication decreasing due to a diminishing pool of activated lymphocytes[12]. To provide definitive evidence that the early CTL response does have an impact on virus replication *in vivo*, we performed an in-depth analysis of CTL-virus interactions at early times post-infection in a patient (WEAU; subject #1 in Clark *et al.* [13]) who presented with acute symptomatic HIV-1 infection. This particular patient was chosen for study because preliminary analysis[2,14] suggested that his early CTL response may have been of limited epitope specificity. In other virus infections, antiviral CTL reach very high frequencies at early times post-infection[15]. We reasoned that if the antiviral CTL response is indeed an important controlling force restricting HIV-1 replication during the acute phase of the infection, then in a patient where the early HIV-1-specific CTL response was directed against a small number of viral epitopes, selection for HIV-1 variants bearing mutations conferring escape from CTL recognition might be observed[16], analogous to viral mutations leading to antiretroviral drug escape.

The results presented in this paper show that CTL directed against a highly immunodominant epitope in the viral glycoprotein were present at extremely high frequency in patient WEAU 16 days after the onset of the acute retroviral syndrome; and that this CTL response was associated with rapid and complete replacement of the plasma virus population with mutant viruses bearing amino acid (aa) changes at a single residue in gp160 which conferred escape from recognition by primary CTL. These findings demonstrate that the early HIV-1-specific CTL response exerts a strong selective pressure on virus replication *in vivo*, and illustrate a mechanism which may contribute to the inability of the antiviral CTL response to achieve more complete control of virus replication during this infection.

Results

Characterization of the very early CTL response in patient WEAU

Patient WEAU was a homosexual male who presented with symptomatic primary HIV-1 infection 20 days following a single sexual encounter with a patient with AIDS, shown by viral DNA sequence analysis to represent the source of infection[13]. Infectious virus was present in the plasma of WEAU at the earliest timepoint tested [15 days following the onset of symptoms (DFOSx)], but the titre declined rapidly, reaching undetectable levels by 23 DFOSx when seroconversion occurred (Table 1). Plasma virion-associated RNA and viral p24 antigen each peaked between 20 and 23 DFOSx. Plasma viral RNA reached its nadir at day 72, and thereafter increased coincident with a decline in CD4 lymphocyte count. Initial analysis of the CTL response mounted by this patient revealed that HIV-1-specific, MHC-restricted $CD8^+$ CTL activity could be detected at the earliest timepoint available for study, 16 DFOSx, and that this early CTL response was directed predominantly against the viral envelope glycoprotein, gp160[2].

To allow a more detailed characterization of the specificity of the primary CTL response, recombinant vaccinia viruses were constructed which expressed full-length gp160 derived from the autologous HIV-1 strain in patient WEAU 15 DFOSx, as well as serial N- and C- terminally truncated sections of this protein. As shown in Figure 1, polyclonal CTL cryopreserved from patient WEAU 20 DFOSx mediated MHC-restricted lysis of target cells expressing the full-length autologous virus gp160 but not other HIV-1 proteins (gag, pol, tat or nef). Within the autologous gp160 protein, all the epitope(s) recognized were located within the N-terminal 110 aa (Figure 1). When a panel of ten gp160-specific MHC-restricted CTL lines/clones derived from patient WEAU 20 DFOSx were screened, they were also all found to

recognize only epitope(s) within aa 1-110 (not shown). To determine the location of these epitope(s), overlapping synthetic peptides corresponding to the N-terminal 110 aa of the day 15 autologous virus gp160 sequence from patient WEAU were used. As representative results in Figure 2a illustrate, all clones tested recognized a single peptide corresponding to gp160 aa 25-41. Using a panel of target cells known to have one or more HLA-A or B alleles in common with patient WEAU (whose HLA type is A1, A29; B8, B44), the restricting HLA molecule for these clones was shown to be HLA-B44 (Figure 2b). A peptide binding motif has been defined for HLA-B44: the anchor residues are E at position 2 and Y or F at position 9, with a second Y or F residue at position 10 forming an auxiliary anchor[17]. A sequence corresponding to this motif was contained in gp160 aa 25-41, at aa 30-39. Peptides of differing lengths based on the gp160 aa 29-39 sequence of patient WEAU's day 15 autologous virus were tested for their relative ability to sensitize autologous target cells for lysis by day 20 WEAU CTL. Both polyclonal CTL and CTL clones were able to recognize a peptide corresponding to gp160 aa 29-39 (Figure 2c and data not shown), confirming that as predicted, the epitope was contained in this sequence. Different CTL clones were found to prefer either aa 30-38 or aa 30-39 as the optimal length of epitope (examples are shown in Figure 2c). Polyclonal CTL from patient WEAU 23 DFOSx recognized both peptides efficiently, giving 50% maximal lysis of target cells presenting each peptide at $5x10^{-6}$ - 10^{-7}M (not shown).

Immunodominance of the gp160 aa 30-38 epitope 16 DFOSx

Limiting dilution analysis indicated that the frequency of CTLp recognizing the gp160 aa 30-38 epitope in recombinant vaccinia virus vM1 was approximately 1 per 17 PBMC 16 DFOSx (not shown). By contrast, the frequency of CTLp recognizing epitopes elsewhere in gp160 (recombinant vaccinia virus vM9) was below the limit of detection in this assay (<1

per 2000 PBMC), as was the frequency of CTLp directed against vSC8, the control vaccinia virus. All detectable HIV-1-specific CTL activity mediated by PBMC cryopreserved from patient WEAU 16 DFOSx was thus directed against a strongly immunodominant viral epitope, gp160 aa 30-38(9). From the very high frequency 16 DFOSx, the frequency of CTLp directed against this epitope subsequently declined to 1 per 49 PBMC 23 DFOSx, and 1 per 7400 PBMC by 136 DFOSx.

Analysis of sequence changes occurring in gp160 in the early stages of the infection

To determine whether the early CTL response mounted by patient WEAU exerted a biologically significant selective pressure on the virus population, we performed a quantitative analysis of sequence changes occurring throughout gp160 over time. Because circulating plasma virus most accurately represents the actively replicating virus population *in vivo*[18,19], we sequenced and compared serial specimens of *uncultured* plasma virus RNA (cDNA). At the initial timepoint (16 DFOSx), plasma virus was essentially homogeneous as assessed by direct population sequencing of virion RNA (cDNA) (example shown in Figure 3a) and by sequence analysis of individually cloned envelope genes (Figure 3b). In other studies (not shown), *gag*, *vpu*, *env*, and *nef* genes were amplified by PCR from *uncultured* PBMC DNA of this patient (10-20 sequences per gene) and found to be similarly homogeneous (<0.5% variation) at this timepoint. Analysis of the *env* gene sequences from plasma virus of patient WEAU between 16 and 136 DFOSx revealed progressive replacement of the initial virus population by one differing primarily at aa 31 (Figure 3). Figure 3a shows that this turnover in the plasma virus population was rapid and complete by day 136. Figure 3b demonstrates further evidence for the strong selective forces acting on this CTL epitope (aa 30-38) since changes within the epitope were numerous and accumulative whereas changes outside it were

less frequent and sporadic. For example, within the CTL epitope, of the 48 *env* clones shown in Figure 3b (boxed area), there were a total of 432 (9aa x 48 clones) potential sites for aa substitution: 37 (8.6%) were changed. In the immediately adjacent sequences shown, there were 2688 (56 aa x 48 clones) potential sites for aa substitution but only 11 (0.4%) changes. Thus, changes within the CTL epitope exceeded by 21-fold those in surrounding areas of *env*. Moreover, the proportion of nonsynonymous nucleotide changes within the CTL epitope (37/38; 97.4%) exceeded that in the surrounding *env* regions (11/16; 68.8%) by 1.4-fold. To further evaluate if the frequency and rapidity of sequence changes observed at positions 30-38 were unique, direct viral RNA (cDNA) population sequencing of the amino-terminal half of gp160 (430 aa) was performed on plasma specimens corresponding to days 16, 30, 44, and 72 DFOSx; the *only* changes observed were those at position 31 (E to G/A). To complete the analysis of HIV-1 quasispecies evolution in this patient, population sequencing of the full-length gp160 (860 aa) from plasma viral RNA (cDNA) from day 136 DFOSx, together with sequence analysis of three full-length gp160 molecular clones derived from the same viral RNA (cDNA) were compared with the viral sequences from day 16. Here, for the first time, changes in addition to those occurring at codon 31 were identified. These included substitutions at aa 356 (K to E), 400 (H to Y), 402 (N to D), 407 (N to D), 815 (V to I), 819 (N to D), and 857 (A to T). (Sequences submitted to GENBANK, accession number XXXXXXX).

Effect of the observed aa changes on epitope recognition by gp160 aa 30-38 -specific CTL

To determine whether the observed changes in the gp160 aa 30-38 epitopic sequence affected CTL recognition of this epitope, synthetic peptides corresponding to aa 30-38 of the predominant mutant virus gp160 sequences were tested for their ability to sensitize autologous

target cells for lysis by polyclonal CTL and CTL clones derived from patient WEAU 16-20 DFOSx. Unlike the wild-type peptide, peptides corresponding to the aa 31 mutant virus sequences which first emerged 30-72 DFOSx and became predominant by 136 DFOSx were not recognized by polyclonal CTL cryopreserved from patient WEAU 16 DFOSx (not shown), or by CTL clones derived from the patient 20 DFOSx (Figure 4). This could either be due to failure of these mutant peptides to bind to HLA-B44, or to lack of recognition of the peptides by CTL following MHC binding: the former seems more likely given that the gp160 aa 31 E residue forms one of the dominant MHC anchor residues in the HLA-B44 peptide binding motif[17]. Interestingly, virus variants with an aa substitution from A to T at position 30 were transiently observed at 44 and 72 DFOSx but were not selected for by 136 DFOSx, and the corresponding peptide was efficiently recognized by epitope-specific CTL (Figure 4). Thus, unlike the early (day 16) or transient (A30T; day 44) viral population, mutants at position 31 (E31G; E31A) were able to escape recognition by the primary CTL response mounted by this patient and accumulated progressively through day 136.

Subsequent evolution of the CTL response in patient WEAU

The emergence of a mutant virus population able to escape recognition by the primary HIV-1-specific CTL response in patient WEAU was not accompanied by a dramatic increase in the plasma viral load in this patient (Table 1). Analysis of the CTL activity mediated by PBMC cryopreserved from patient WEAU 30 DFOSx (Figure 5) revealed that even by this relatively early timepoint responses were detectable to several new viral epitopes, including at least one in gp160 aa 111-860, plus epitopes in gag, pol and nef. CTL of these novel specificities would be capable of recognizing their antigens associated with the gp160 aa 31 mutant virus population, and thus could account for its continued containment.

Discussion

Here we provide evidence that the early HIV-1-specific CTL response exerts a substantial controlling pressure on virus replication *in vivo*. The present study is unique in that it represents a detailed molecular analysis of CTL-virus interactions very early after HIV-1 infection, prior to seroconversion and even before viral RNA had achieved peak titers. The first timepoint analyzed, 15 DFOSx, was exactly 35 days after the patient was infected by HIV-1[13]. The kinetics of decline in plasma viremia coincided temporally with the appearance, first of a strong CTL response focused on a highly immunodominant epitope at gp160 aa 30-38, and shortly thereafter, of broader CTL reactivity. Evidence for a strong and biologically important selective pressure exerted by CTL was provided by the observation that by 30-44 DFOSx, CTL escape mutants were detectable. By 136 DFOSx, there had been complete replacement of the transmitted virus strain (which had initially replicated to high titers) by a mutant population that differed primarily at aa 31. The cellular compartments harboring replicating virus early in infection were thus largely eliminated and replaced by cells infected with CTL escape variants by this timepoint. The magnitude of wild-type virus decline, the kinetics of mutant virus appearance, and the genetic pathways by which virus escaped CTL recognition, bear certain similarities to viral dynamics in the setting of antiretroviral drug therapy. For example, between 16 and 72 DFOSx, wild-type virus in the plasma (defined by aa 30-38 sequence AENLWVTVY) declined from 200,000-300,000 RNA molecules per milliliter to approximately 1,000 (10% of 11,400). Mutant virus first appeared at 30 DFOSx and evolved in a complex pattern until a best-fit population not recognized by HLA B44-restricted CTL came to predominate at day 136. Viral evolution to the aa 30-38 escape variant

would likely have occurred even more rapidly, and plasma virus titers reached higher levels, had not CTL responses directed against other viral gene products developed.

HIV-1 variants which are not recognized by autologous CTL have previously been observed in longitudinal studies of HIV seropositive patients[21-23]; but it has generally been difficult to demonstrate that CTL escape mutant viruses have a clear selective advantage *in vivo*. One factor that may have facilitated the demonstration of CTL escape variants in this study may be our approach for identifying changes in the viral quasispecies by analyzing plasma viral RNA (cDNA). Plasma virus exhibits a circulating half-life ($T_{½}$) of approximately six hours[20] and the cells producing most of this virus a $T_{½}$ of approximately 2 days[18,19]. However, these virus-producing cells are under-represented in the blood where latently-infected cells and defectively-infected cells (with half-lives as long as 80 days) predominante (Shaw *et al.* unpublished). Analysis of plasma viral RNA gives a dynamic assessment of the most active viral compartments.

Immunologically, the fact that the earliest CTL response in patient WEAU was predominantly focused on a highly immunodominant viral epitope and that the CTLp frequency was so high early after infection also may explain why CTL-mediated selection of escape-conferring mutations was more evident in this patient than in previous studies[21-25]. If CTL pressure is simultaneously directed against several codominant epitopes, the outgrowth of virus variants with escape-conferring mutations in only one of these epitopes may be controlled by CTL directed against the other epitopes[26,27] (unless the variant epitopes have strongly antagonistic properties)[28]. At 16 DFOSx, limiting dilution assays showed the frequency of epitope-specific CTLp to be approximately 1 per 17 PBMC and even this value is likely to be an underestimate since the PBMC had been cryopreserved prior to testing. This frequency is

similar to the CTLp frequencies measured at the peak of the acute response to LCMV infection in mice[15,29]. The primary CTL response in patient WEAU may thus have been near its peak around 16 DFOSx. Whether HIV-1-specific CTLp generally reach such high frequencies at the peak of the early immune response is currently unclear.

As patient WEAU obviously had the capacity to mount CTL responses to multiple HIV-1 epitopes, it remains unclear why the earliest CTL response in this patient was so predominantly focused against the gp160 aa 30-38(9) epitope. One possibility is that prior unrelated infections in this patient left him with a population of memory T cells which crossreacted on this epitope and that these cells, being present at higher frequency and more readily activated than naive T cells directed against other HIV-1 epitopes, dominated the initial HIV-1-specific immune response. That the CTL response mounted to a virus infection may be modified by the host's prior immune experience with unrelated pathogens has been demonstrated in murine virus infections[30]. Alternatively (or in addition), the gp160 aa 30-38(9) epitope may have been particularly immunogenic. In this context, it is of interest that during the natural processing of gp160, signal peptide cleavage occurs between aa 29 and 30, thus generating the same N-terminus as the aa 30-38 CTL epitope. This may have favored production of the aa 30-38 peptide at higher levels than other epitopic peptides, and resulted in preferential presentation of this epitope especially at very early times after infection when levels of viral antigens were limiting. The immunogenicity of a peptide is also influenced by its affinity of binding to MHC and the affinity of peptide-MHC complexes for the T cell receptor. The gp160 aa 30-38(9) epitope may have been "strong" in these respects, and the epitopes recognized later may have been "weaker". The shift in the response to "weaker" epitopes may have reduced the long-term efficiency of containment of virus replication[23] and

been one of the factors which contributed to the subsequent rapid rate of disease progression in patient WEAU[31]. Recent studies[32] suggest that the viral load established early in HIV infection is a predictor of the subsequent clinical course, with higher viral loads after seroconversion predicting shorter survival. The shift in CTL specificity which occurred around the time of seroconversion in patient WEAU may also have influenced the viral "setpoint" established in this patient, again contributing to the rapid rate of disease progression he underwent.

Future in-depth analysis of virus-CTL interactions in larger numbers of patients is needed to reveal how commonly CTL escape virus variants are selected for during acute HIV-1 infection, and whether their selection is correlated with the establishment of a high viral "setpoint" and a rapid rate of subsequent disease progression. Preliminary results we have obtained in a second patient show that here too, rapid selection occurred within the plasma virus population for mutants bearing aa changes in a gp160 epitope recognized by the early CTL response. Interestingly, this patient was also a rapid disease progressor. The events we report here in patient WEAU are thus not unique and may in fact prove to represent a mechanism that HIV-1 commonly uses to evade control by the early antiviral immune response.

In summary, the results presented here provide the first direct demonstration of the substantial and biologically relevant pressure exerted by the early $CD8^+$ CTL response on HIV-1 replication *in vivo*. Together with the large body of suggestive evidence already in the literature that virus-specific $CD8^+$ CTL make an important contribution to containment of virus replication during infection with HIV-1[5,6,8-11], these findings suggest that stimulation of this arm of the antiviral immune response should be an important goal of future prophylactic and therapeutic strategies to combat HIV-1 infection.

Methods

Patient samples

The clinical profile of patient WEAU has been reported[13]. After obtaining informed consent, blood specimens were collected in acid-citrate-dextrose. PBMC were isolated and cryopreserved as previously described[2]; plasma was also cryopreserved at each timepoint.

Recombinant vaccinia viruses

Recombinant vaccinia viruses vSC8, which expresses only *Escherichia coli* β-galactosidase; vPE16, which expresses gp160 from HIV-1 IIIB; vCF21, which expresses Pol from HIV-1 HxB2; vTFnef2, which expresses Nef from HIV-1 NL43 and vtat, which expresses Tat from HIV-1 IIIB were obtained from Dr. Bernard Moss (NIH, Bethesda, MD); and recombinant vaccinia virus vAbT 141-5-1, which expresses the full-length p55 Gag protein from HIV-1 IIIB[33] was obtained from Drs. Dennis Panicali and Gail Mazzara (Therion Biologics Corporation, Cambridge, MA).

Recombinant vaccinia viruses expressing full-length gp160 (vM12) or sections thereof (vM1 and vM9) derived from the autologous HIV-1 in patient WEAU 15 DFOSx were produced by homologous recombination into the thymidine kinase gene of vaccinia virus using the vaccinia transfer plasmid pNVV3, a modified version of pSC11, as described[34]. The clone expressing full-length gp160 used for the construction of the recombinant vaccinia viruses was obtained from a full-length replication-competent proviral clone (1.60) derived by lambda phage cloning of an isolate obtained 15 DFOSx[35]. The envelope gene was subcloned into plasmid pCR II (Invitrogen Corporation, San Diego, CA), from which it was subsequently excised and ligated into the Sma 1 and Not 1 sites of pNVV3 to yield pM12. Sections

corresponding to nucleotides (ntd) 1-330 and 334-2580 of the *env* gene were amplified from the full-length clone by PCR using oligonucleotide primers which incorporated the necessary start and stop codons as well as a Sma 1 site at the 5′ end and a Not 1 site at the 3′ end. The PCR products were then ligated into pNVV3 to yield pM1 (*env* ntd 1-330) and pM9 (*env* ntd 334-2580). pM12, pM1 and pM9 were used to produce recombinant vaccinia viruses vM12, vM1 and vM9, respectively. Protein expression was confirmed by Western blotting (not shown).

CTL assays

In some assays the effector cells were polyclonal patient CTL. Here, cryopreserved PBMC were expanded *in vitro* by bulk culture for 10 days as previously described[2]. For use in other experiments, CTL lines/clones were established from PBMC cryopreserved from patient WEAU 20 DFOSx by culture at limiting dilution as described[36]; these are operationally referred to as clones. CTL activity was assayed in a conventional 5-hour ^{51}Cr release assay as described[2]. Target cells were autologous (HLA A1, A29; B8, B44) and allogeneic EBV-B-LCL, either uninfected or infected at a multiplicity of infection of 10 plaque forming units per cell 16 hours previously with different recombinant vaccinia viruses. In some assays, synthetic peptides were added to the assay medium at different concentrations. Peptides were synthesized by the solid-phase method on an automated peptide synthesizer with FMOC chemistry, purified, and their identity confirmed as described[37]. CTL assay results are expressed as the % specific ^{51}Cr release, calculated as 100 x (experimental release-spontaneous release)/(maximum release-spontaneous release).

Limiting dilution analysis of specific CTLp frequency

Precursor frequencies of specific CTL were estimated by limiting dilution analysis as described[33]. Cryopreserved patient PBMC were plated at a range of dilutions, and were restimulated by *in vitro* culture in IL-2-containing medium with an anti-CD3 antibody and irradiated allogeneic PBMC from a normal donor to allow expansion of CTL. Wells were then split and assayed for cytotoxicity on ^{51}Cr-labeled autologous target cells infected with different recombinant vaccinia viruses or coated with synthetic peptide AENLWVTVY. The fraction of non-responding wells was calculated by determining the number of wells in which ^{51}Cr release did not exceed 10% specific lysis. Precursor frequencies were then estimated by single hit model Poisson distribution analysis[38].

Gp160 sequence analysis

The gp160 sequence of the autologous virus from patient WEAU 15 DFOSx was determined by automated DNA sequencing of the WEAU 1.60 provirus and is reported as the HIV-1 reference sequence in the 1995 HIV Molecular Immunology Database[35]. Quantitative detection of gp160 mutations occurring in the uncultured plasma virus population over time was performed as described[18]. HIV-1 RNA was isolated from virions pelleted from plasma specimens, and cDNA prepared using antisense oligonucleotide primers corresponding to either 5'-TTGCTACTTGT GATTGCTCCATGT-3' (nt 8920-8943), 5'-TCTTATGAGTGTGGTGACATTGAAAGA-3' (nt6706-6732), or 5'-CAGAGTGGGGTTAATTTTACAC ATGG-3' (nt 6571-6596) (numbering according to HIV-1 proviral clone WEAU 1.60[39]). Full length and partial gp160 gene sequences were amplified by nested PCR as described[18] using the primers listed above along with primers at positions

5851-5875, 5956-5981, 6203-6326, 6436-6455, 6571-6596, and 8889-8911. Primers incorporated the universal M13 sequence for subsequent dye-primer sequence analysis of the gp160 aa 30-39 region. For sequence analysis of the complete gp160 gene, dye-labeled dideoxynucleotide terminators were used. A total of 4-6 separate PCR amplifications of plasma viral RNA/cDNA (750 molecules/reaction) was done for each timepoint. Double strand sequence analysis was performed using an automated ABI 373A sequenator and Dye Primer/Dye Terminator Cycle Sequencing Kits (ABI). Sequences were analyzed using Sequencher (Gene Codes Corp.) and Microgenie (Beckman) software packages, and base-pair mixtures were quantitated by measuring relative peak-on-peak heights[18]. Relative proportions of wild-type and mutant sequences were determined independently by subcloning the amplified gp160 envelope products (above) into pCR3 (Invitrogen Corporation, San Diego, CA) and subjecting the individual clones to double-stranded automated DNA sequencing.

Acknowledgments

We are grateful to Dr. Bernard Moss for providing us with recombinant vaccinia viruses vSC8, vPE16, vCF21, vTFnef2 and vtat; Drs. Dennis Panicali and Gail Mazzara for recombinant vaccinia virus vAbT 141-5-1 and Dr. J. Lindsay Whitton for plasmid pNVV3. We thank Dr. Honoré Mazarguil for peptide synthesis, Diana Frye for outstanding secretarial assistance, and Dr. David Tough for critical appraisal of the manuscript.

This work was supported by grants from the NIH, NIAID AI37430 (PB), AI35467 (GMS), AI09484 (MBAO; JEG) and MH51519 (JAN); the HIV Correlates of Human Immune Protection Contract NO1-AI45218 (GMS; BHH); and by the core research facilities of the University of Alabama Center for AIDS Research (AI27767) and Birmingham Veterans Administration Medical Center.

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Figure 1. All detectable HIV-1-specific CTL activity mediated by PBMC cryopreserved from patient WEAU 20 DFOSx is directed against epitope(s) in the first 110 aa of gp160. The results shown are the % specific ^{51}Cr release mediated by *in vitro* restimulated polyclonal CTL at an effector : target cell (E:T) ratio of 40:1 from autologous and allogeneic EBV-B-LCL target cells infected with recombinant vaccinia viruses encoding β-galactosidase (b-gal) only (vSC8); full-length gp160 (vM12) or sections thereof (vM1 and vM9) derived from the autologous virus in this patient 15 DFOSx; or other HIV-1 proteins (vAbT 141-5-1, vCF21, vTFnef2 and vtat) as indicated.

Figure 2. Epitope mapping studies showing that CTL clones derived from patient WEAU 20 DFOSx recognize gp160 aa 30-38(9) in association with HLA-B44. *a*, % specific ^{51}Cr release mediated by two CTL clones at an E:T ratio of 10:1 from autologous EBV-B-LCL target cells in the presence of 100μg/ml of a series of synthetic peptides (each 17 aa long and overlapping by 5aa) corresponding to the gp160 sequence of the autologous virus of patient WEAU 15 DFOSx. The aa 25-41 peptide recognized by both clones (and others tested) has the sequence MICSAAENLWVTVYYGV. *b*, lysis mediated by CTL clone #10-4 at an E:T ratio of 10:1 of autologous EBV-B-LCL target cells (HLA-A1, A29; B8, B44) and EBV-B-LCL sharing between 0 and 3 HLA-A or B molecules with patient WEAU as indicated, following infection with recombinant vaccinia viruses vM1 (expresses autologous gp160 aa 1-110) or vSC8 (expresses b-gal only). Target cells sharing HLA-B44 with patient WEAU were recognized by this and other day 20 WEAU CTL clones (not shown) after infection with vM1. *c*, % specific ^{51}Cr release mediated by two CTL clones at an E:T ratio of 10:1 from autologous target cells in the presence of different concentrations of the synthetic peptides

shown, which represent the gp160 aa 29-39 sequence of the autologous virus of patient WEAU 15 DFOSx (AAENLWVTVYY), and amino or carboxy-terminal truncated versions of this peptide. The optimal epitope length for recognition by the two clones differs, clone #5-1 preferring (A)AENLWVTVY, whilst clone #10-9 prefers (A)AENLWVTVYY.

Figure 3. Quantitative detection of HIV-1 CTL escape mutations by automated sequence analysis of plasma viral RNA (cDNA). *a*, population sequences of codons 30-34 of the gp160 gene are shown, demonstrating the emergence of mutant virus [GAA(E) to GAA/GGA (E/G) at codon 31] on day 44 and with complete replacement of wild-type virus (AENLW) by mutant virus (AGNLW/AANLW) by day 136. *b*, deduced amino acid sequences of individual plasma viral RNA (cDNA) clones corresponding to the amino-terminal 65 aa of gp160. The CTL epitope at position 30-38 is boxed.

Figure 4. Relative abilities of synthetic peptides corresponding to gp160 aa 30-38 of the virus population in patient WEAU 16 DFOSx, and of the predominant mutant populations which emerged later, to sensitize autologous target cells for lysis by early WEAU CTL. The results shown are the % specific ^{51}Cr release mediated by day 20 WEAU CTL clone #5-1 at an E:T ratio of 10:1 from autologous EBV-B-LCL target cells in the presence of different concentrations of synthetic peptides, as indicated.

Figure 5. PBMC cryopreserved from patient WEAU 30 DFOSx mediate CTL activity directed against multiple HIV-1 epitopes. The results shown are the % specific ^{51}Cr release mediated by *in vitro* restimulated polyclonal CTL at an E:T ratio of 40:1 from autologous and allogeneic EBV-B-LCL target cells infected with recombinant vaccinia viruses encoding b-gal only (vSC8); gp160 derived from the plasma virus in patient WEAU 15 DFOSx and sections

Table 1. Clinical Course of Patient WEAU

		HIV-1 Antibody■		Plasma Viremia+		
DFOSx*	CD4	ELISA	WB	Infectivity	RNA	p24 Ag
15	358	-	-	1000	216,400	80
16		-	-	1000		103
20		+	-	1	355,200	299
23		+	+	0	355,400	258
27	748	+	+	0	146,800	32
34		+	+	0	100,900	11
44	972	+	+	0	34,700	0
72		+	+	0	11,400	0
136		+	+	0	17,322	0
212	197	+	+	0	90,109	0
391	89^	+	+	5	55,268	0

*DFOSx - days following onset of symptoms of the acute retroviral syndrome.

■Presence (+) or absence (-) of HIV-1-specific antibody as determined by enzyme-linked immunosorbent assay (ELISA) or Western Blot (WB)[13].

+Plasma viremia as determined by infectivity titers (tissue culture infectious doses per milliliter of plasma[13]); plasma viral RNA (molecules per milliliter[31]); and HIV-1 p24 core antigen after antigen-antibody dissociation (pg per milliliter[31]).

^CD4 counts declined to 30/mm^3 772 DFOSx and to 51mm^3 1099 DFOSx. The patient received antiretroviral therapy beginning 540 DFOSx but died 1601 DFOSx.

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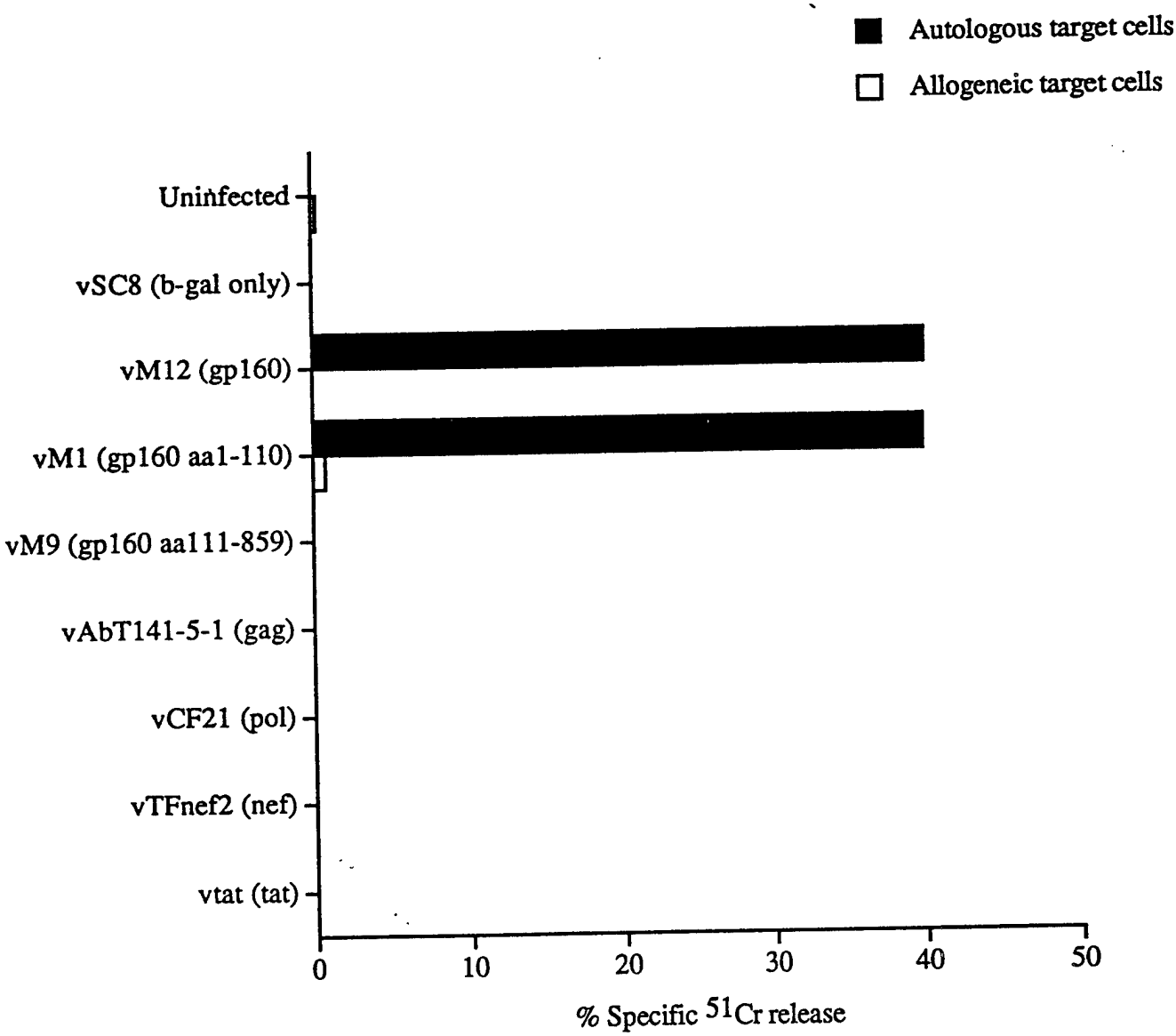
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Allogeneic target cells
Uninfected
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vM1 (gp160 aa1-110)
vM9 (gp160 aa111-859)
vAbT141-5-1 (gag)
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vTFnef2 (nef)
vtat (tat)
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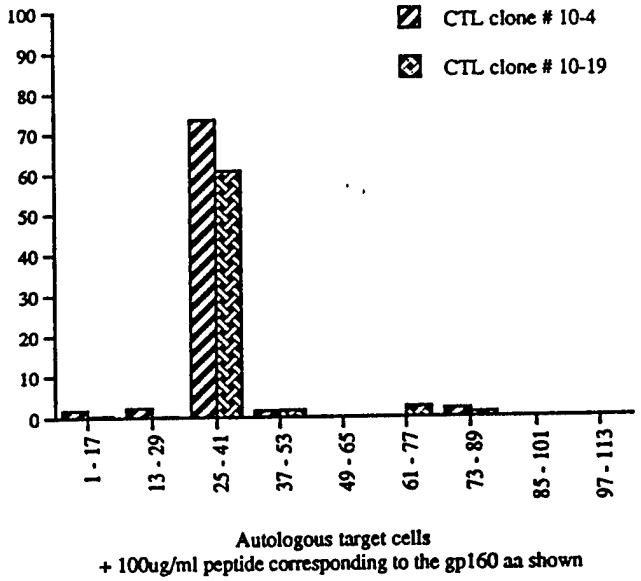
10
20
30
40
50
% Specific 51Cr release

a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30
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10
0
1 - 17
13 - 29
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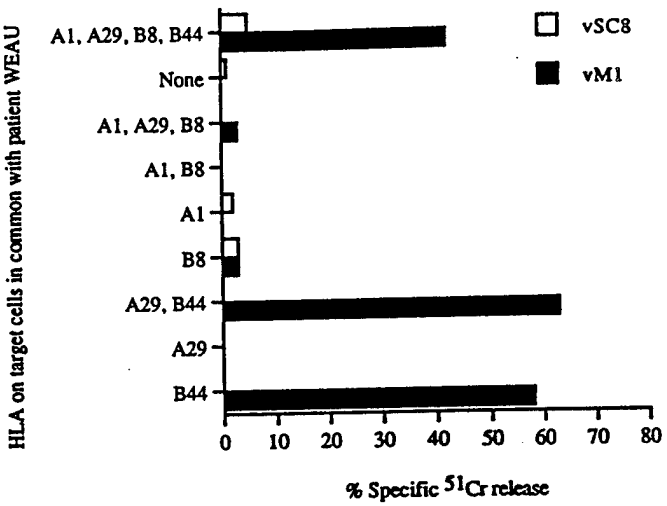
49 - 65
61 - 77
73 - 89
85 - 101
97 - 113
Autologous target cells
+ 100ug/ml peptide corresponding to the gp160 aa shown

b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B44
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vM1
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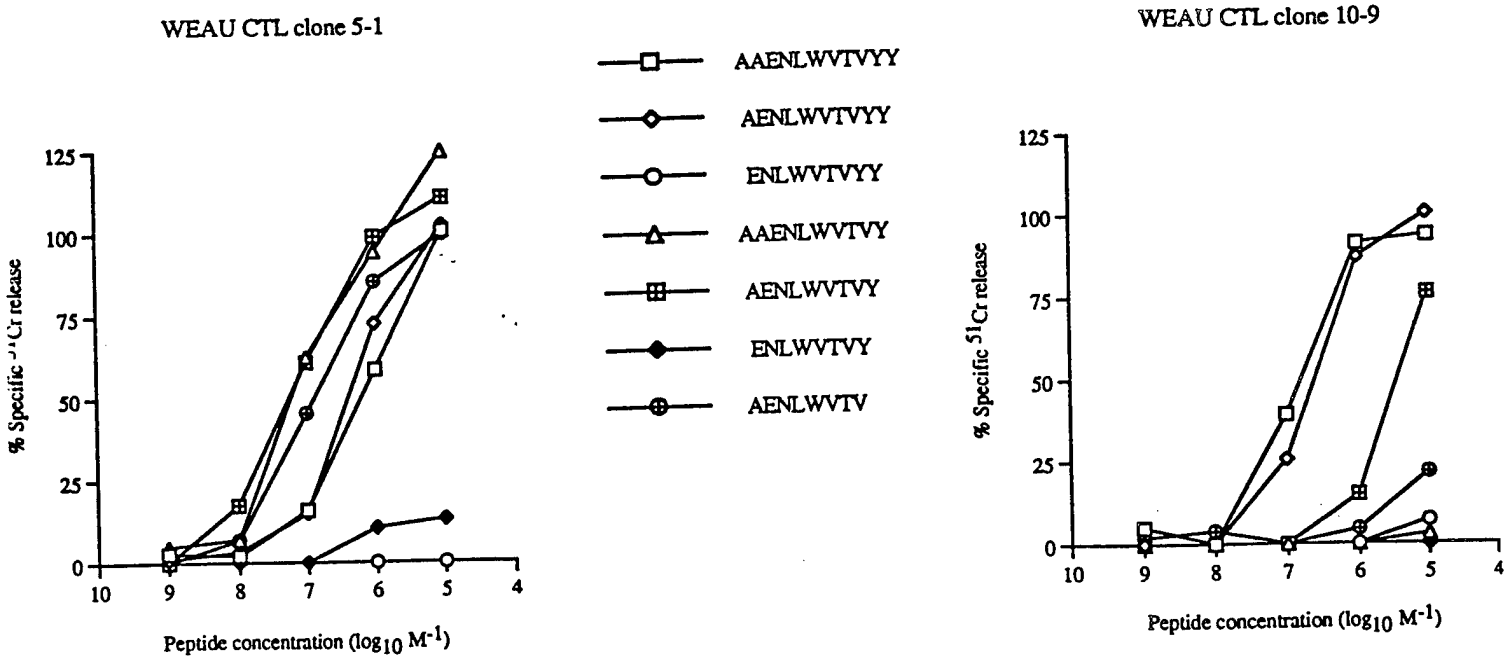
% Specific 51Cr release

c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AENLWVTV
% Specific 51Cr release
125
100
75
50
25
0
10 9 8 7 6 5 4
Peptide concentration (log10 M-1)
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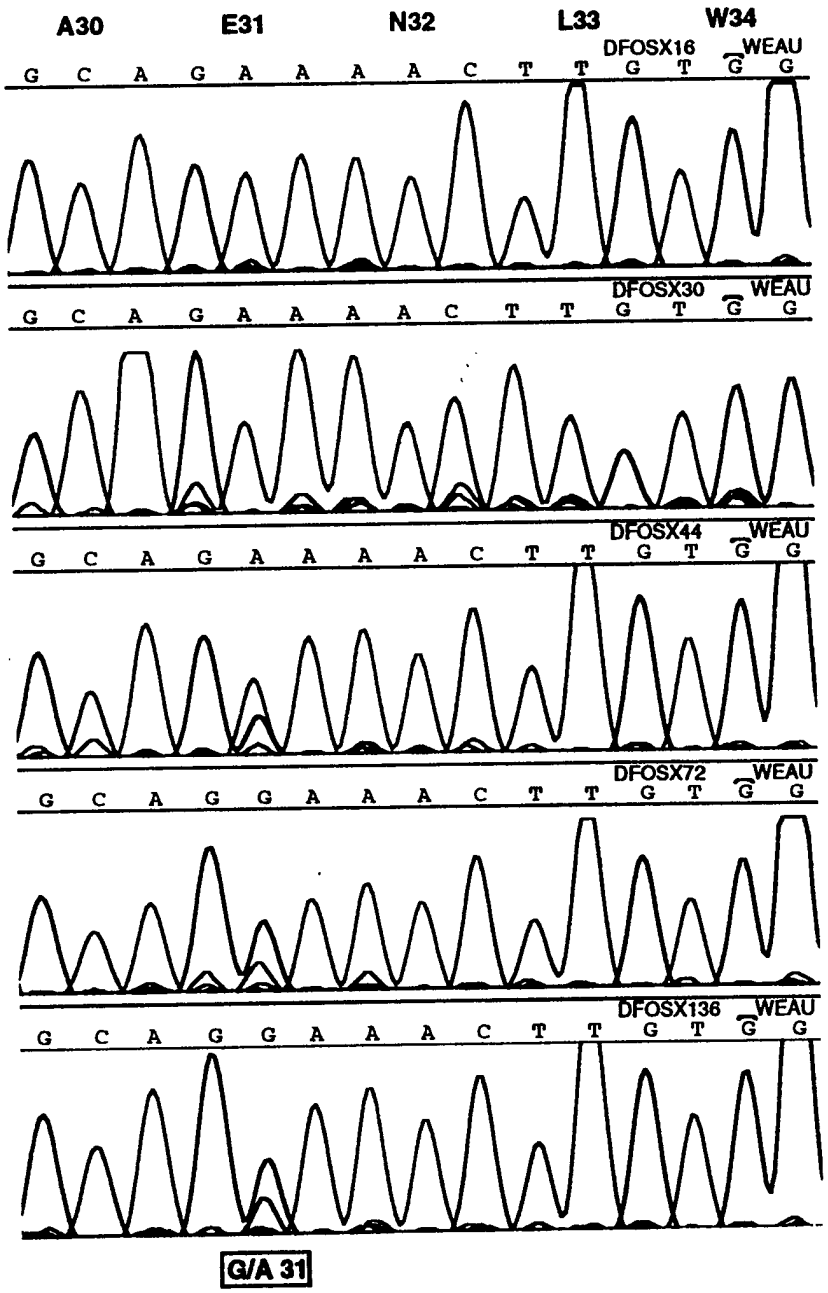
10 9 8 7 6 5 4
Peptide concentration (log10 M-1)

a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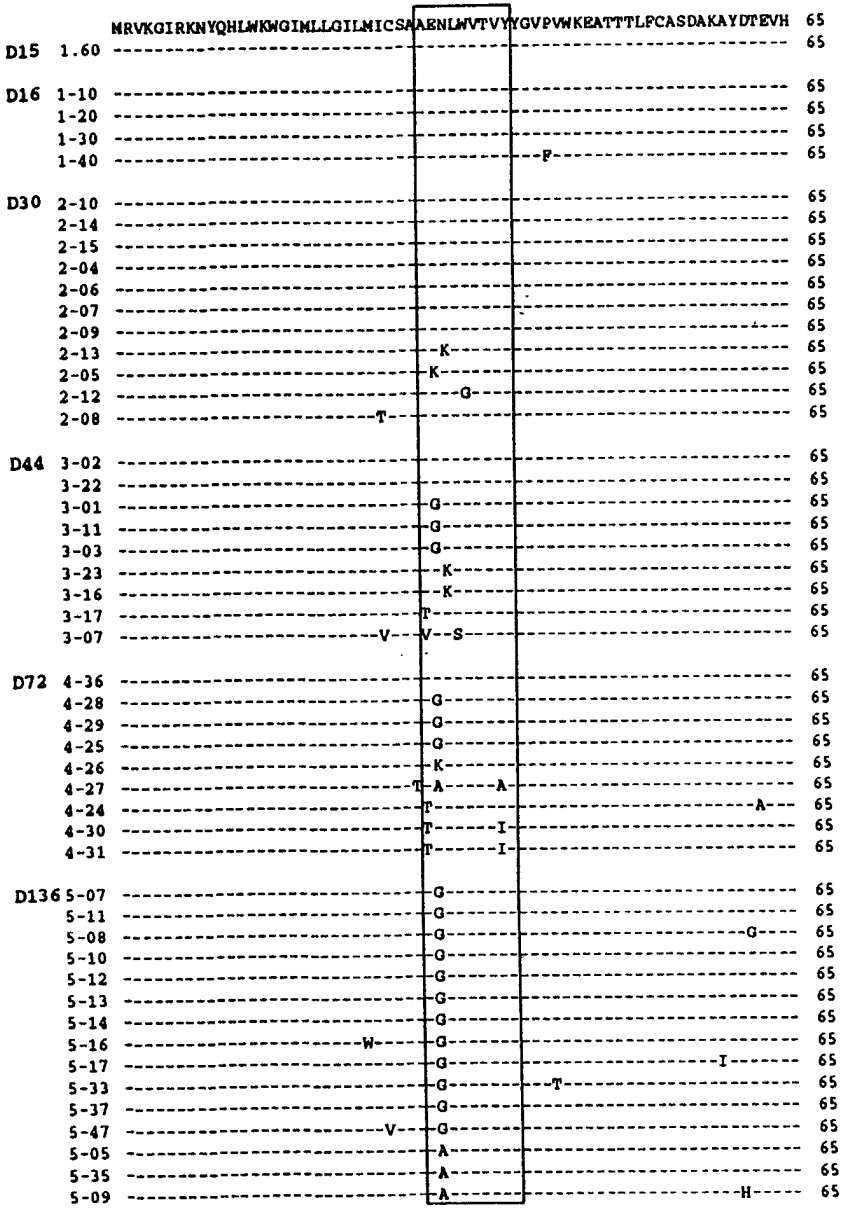
b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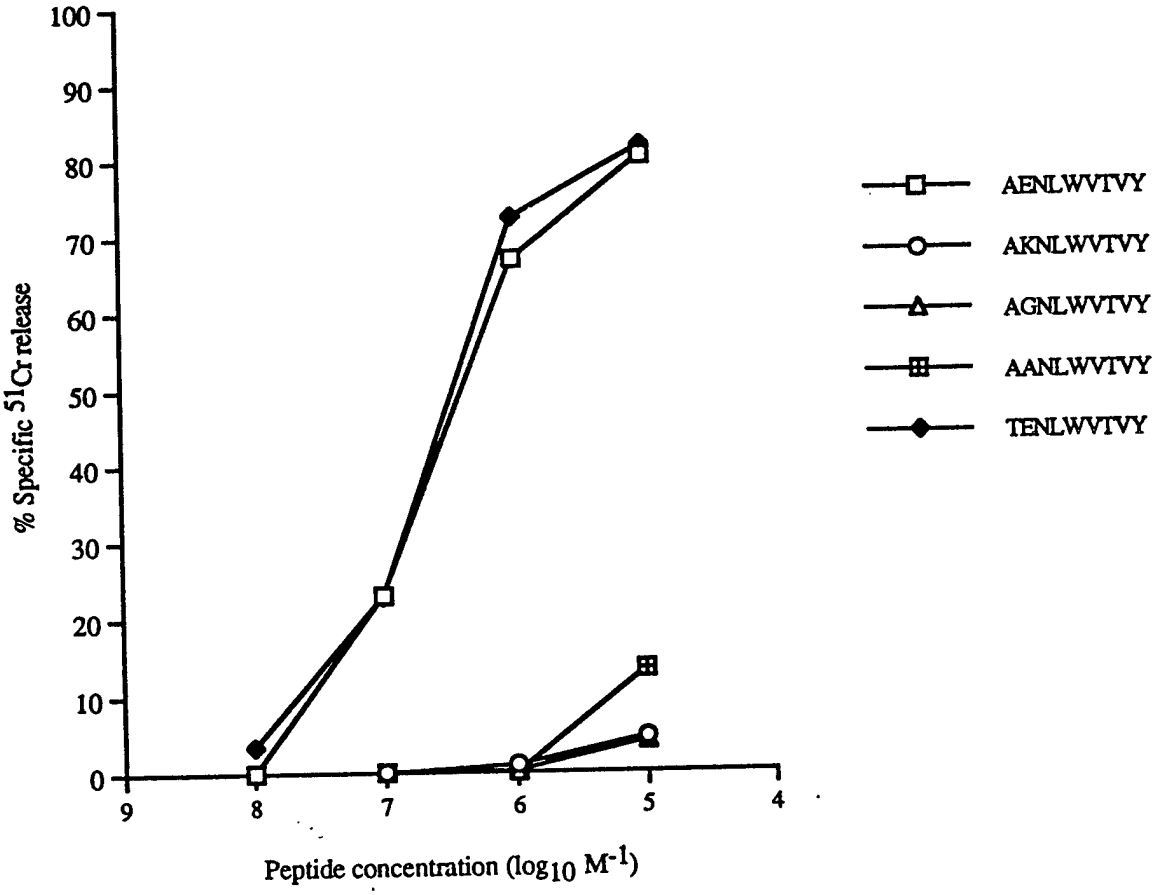
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90
80
70
60
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40
30
20
10
0
% Specific 51Cr release
9
8
7
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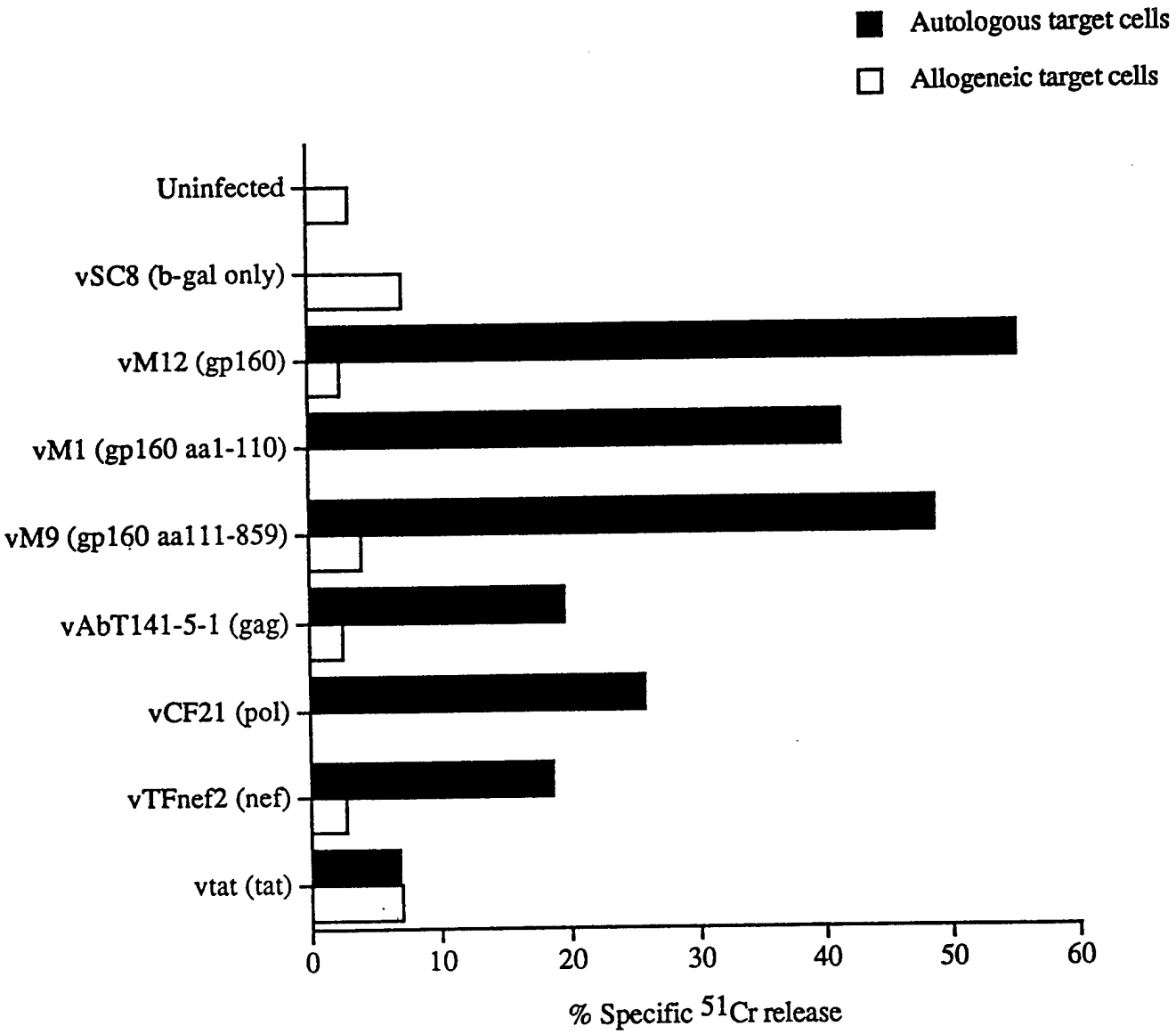
Autologous target cells
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vSC8 (b-gal only)
vM12 (gp160)
vM1 (gp160 aa1-110)
vM9 (gp160 aa111-859)
vAbT141-5-1 (gag)
vCF21 (pol)
vTFnef2 (nef)
vtat (tat)
0
10
20
30
40
50
60
% Specific 51Cr release

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SAMPLE ABSTRACT

DRUG RESISTANCE IN THE CONTEXT OF THE THREE-DIMENSIONAL STRUCTURE OF HIV-1 REVERSE TRANSCRIPTASE

A. Jacobo-Molina, J. Ding, R.G. Nanni, X. Lu, C. Tantillo, A.D. Clark, Jr., P. Boyer*, S. Hughes* and E. Arnold. Center for Advanced Biotechnology and Medicine and Rutgers University, Piscataway, NJ, USA; *NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA.

The structure of HIV-1 reverse transcriptase (RT), is expected to accelerate our understanding of drug resistance against both nucleoside and nonnucleoside inhibitors. Last year Kohlstaedt *et al.* reported the .....

QUANTITATIVE ANALYSIS OF REPLICATION-ACTIVE, LATENT, AND DEFECTIVE FORMS OF HIV-1 PROVIRUS IN PERIPHERAL BLOOD: IMPLICATIONS FOR VIRAL PATHOGENESIS AND THERAPY.

R. Taylor[1], J. Germany-Decker[1], X. Wei[1], J. Lifson[2], M. Nowak[3], M. Saag[1], B. Hahn[1], and G. Shaw[1]. [1]University of Alabama at Birmingham, Birmingham, AL, USA; [2]Genelabs, Inc., Redwood City, CA; [3]Oxford University, Oxford, UK.

HIV-1 load *in vivo* is comprised of cell-free virus as well as substantial numbers of replication-active, latent, or defective viral genomes, all of which likely play a role in disease pathogenesis. Recent studies have emphasized the dynamics of virus turnover in plasma and the clinical benefits associated with decreases in plasma viremia following antiretroviral therapy. Yet, antiretroviral regimens which maximally impact all viral "compartments" are likely to result in the greatest therapeutic gains and the longest delay in the development of drug resistance. It is thus essential to elucidate the numbers and half-lives of cell populations harboring active, latent, or defective viral forms. We utilized a combination of biologic (phenotypic) and genetic approaches to quantify these viral forms in peripheral blood mononuclear cells (PBMCs). Three subjects (CD4+ cells 20-200/mm^3) were studied before and 14, 28, 42, and 140 days after initiation of Nevirapine (NVP) antiretroviral therapy. At baseline, the total viral DNA content of PBMCs determined by QC-PCR ranged from 450-800 molecules per 10^6 cells. Infectivity titers determined by biological cloning ranged from 17-29 infectious units per 10^6 total cells. After initiation of NVP therapy, infectious PBMC-associated virus titers fell significantly more (30-fold) than did the total number of HIV-1 DNA positive cells (2-fold). Clearance rates of specific virally-infected cell populations were determined by quantifying the elimination of wild-type virus and its replacement by drug-resistant mutant virus. The half-life of cells infected with total virus (defective plus competent) determined by direct population sequencing and in situ RT expression was approximately 50-100 days. The half-life of cells harboring replication-competent virus determined by biological cloning and phenotypic resistance testing was biphasic, reflecting separate cell populations containing either transcriptionally-active or latent virus infection: approximately 90% of PBMCs containing replication-competent virus were eliminated within 14 days ($T1/2 < 5$ days), whereas another 1-10% of such cells persisted for at least 42 days. The results of these studies thus define three populations of virally-infected PBMCs. The largest population (approximately 450-800 cells per million) contains largely defective virus and exhibits a half-life of 50-100 days; a second population, much smaller (17-29 cells per million), contains actively replicating virus and is eliminated with a half-life of < 5 days, similar to lymphoreticular cells responsible for sustaining plasma viremia (*Nature* 373:117-126, 1995); a third population, still smaller (approximately 1 cell per 3.3 million), contains latent virus and exhibits a lifespan of at least 42 days. These findings indicate that although PBMCs harbor a predominately archival record of prior (abortive) viral infection, they also contain replication-competent genomes in both transcriptionally-active and latent states. These results provide a scientific framework with which to extend studies of viral and cellular dynamics into the lymphoreticular tissue compartments.

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